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(54) Title: METHOD OF INHIBITING REDUCTION OF DISULFIDE BONDS

(57) Abstract

This invention pertains to a method of altering (inhibiting or enhancing), directly or indirectly, reduction of disulfide bonds of membrane-bound macromolecules, particularly proteins, and, as a result, inhibiting (totally or partially) or enhancing the cellular penetration and the respective effects of these macromolecules. In particular, the present invention relates to a method of inhibiting, directly or indirectly, the reductive function of cell membranes, particularly the cell surface (plasma) membrane, which is capable of cleaving disulfide bonds in membrane-bound proteins which must be cleaved for the proteins to enter cells and produce their respective effects on cells they have entered. Cleavage of disulfide bonds in this manner is a metabolic step required for the ultimate function of the protein or macromolecule. As described herein, applicant has demonstrated that the reductive function of cell surface membranes is catalyzed by protein disulfide isomerase (PDI). The method of the present invention is useful to prevent adverse effects of toxins, such as diphtheria toxin, on cells. It is also useful in preventing infection (e.g., viral or bacterial) or the spread of an established infection. The method may also be useful in enhancing uptake of macromolecules whose presence within a cell is desired.

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METHOD OF INHIBITING REDUCTION OF DISULFIDE BONDS

BACKGROUND OF THE INVENTION

Entry of many toxins and viruses into cells is driven by receptor-mediated endocytosis, in which a specific receptor protein on the membrane surface recognizes an extracellular macromolecule (ligand) and binds to it. Subsequently, this region of the plasma membrane, which includes the ligand bound to the surface receptor, invaginates, forming an endosome, which is a new intracellular membrane-bound vesicle completely surrounded by the cell cytoplasm. The endosome undergoes acidification which in many instances causes the receptor and ligand to dissociate. The receptor is recycled back to the plasma membrane; the ligand often becomes trapped by intracellular vesicles called lysosomes which contain enzymes that degrade the ligand.

Cleavage of disulfide bonds is a necessary step in the activation of a variety of endocytosed macromolecules, such as the toxins diphtheria toxin, ricin, abrin, modeccin, pseudomonas endotoxin, cholera toxin and tetanus toxin, which are made of two chains held together by disulfide bonds. A well-characterized example of this process is the translocation of the diphtheria toxin, secreted by the Corynebacterium Diphtheriae, across the membrane of early endosomes. Diphtheria toxin, which kills cells by irreversibly inhibiting protein synthesis, is secreted as a single-chain precursor comprised of 2 polypeptide chains, A and B, which are linked by a disulfide bond. It is currently believed both chains of the diphtheria toxin are internalized within the cell by receptor-mediated endocytosis. It is also believed that the B chain of nicked diphtheria toxin undergoes conformational changes in acidic endosomes and inserts itself into the endosomal membrane to facilitate

translocation of the A chain into the cytoplasm, where inhibition of protein synthesis occurs. It is assumed that prior to translocation of the A chain, reduction of the interchain disulfide bond occurs, which allows the two chains to separate. However, little is known about the site or mechanism of this reductive cleavage, except that in the case of diptheria toxin it must occur very soon after binding of the toxin to its surface receptor.

Just as the chain B of diphtheria toxin directs the chain A to the endocytic pathway, other macromolecule such as alpha-2 macroglobulins are known to act as carriers of endocytosis for other proteins, such as enzymes and peptide hormones and they do so by linking themselves by disulfide bond(s) to the macromolecule to be carried. Such linkage would also be expected to be cleaved by a cell-surface associated reductive mechanism.

Although the importance of cleavage of disulfide bonds for activation or entry into cells of many macromolecules is known, little is known about the mechanism by which it occurs and the molecules or cellular components which participate. More knowledge is required to assess whether this process can be modulated either to enhance the cellular entry of macromolecules which are beneficial to cells (e.g., hormone, growth factors) or to inhibit the penetration of macromolecules or macromolecular compounds which are detrimental to cells (e.g., toxin, virus).

SUMMARY OF THE INVENTION

This invention pertains to a method of altering (inhibiting or enhancing), directly or indirectly, reduction of disulfide bonds in the binding area of specific macromolecular ligands, such as membrane-bound molecules or proteins, and, as a result, inhibiting (totally or partially) or enhancing the cellular penetration

and the respective effects of all or part of these macromolecules. In particular, the present invention relates to a method of inhibiting, directly or indirectly, the reductive function of cell membranes, particularly the cell surface (plasma) membrane, which is capable of cleaving disulfide bonds in the binding area of specific protein ligands which must be cleaved for all or part of the proteins to enter cells and produce their respective effects on cells they have entered. Cleavage of disulfide bonds in this manner is a metabolic step required for the ultimate function of the protein or macromolecule. As described herein, Applicant has demonstrated that the reductive function of cell surface membranes is catalyzed by protein disulfide isomerase (PDI, EC5.3.4.1).

In one embodiment, the present invention is a method of inhibiting, directly or indirectly, the function of protein disulfide isomerase (PDI), which catalyzes cleavage of disulfide bonds in membrane-bound macromolecules which must be cleaved for passage of all or part of the protein across the cell membrane and, thus, for the protein to have its effect on the cell. In the case in which uptake of the macromolecule is inhibited, the reductive function of the cell membrane is inhibited, directly, by inhibiting the reductive function of PDI and thus decreasing the rate at which disulfide bonds in membrane-bound macromolecules are cleaved, or indirectly, by altering the configuration or structure of the membrane-bound macromolecule to render the disulfide bonds which must be reductively cleaved for uptake of the macromolecule less available to the reductive function of the cell-surface membrane. The macromolecule is therefore taken up into the cell to a lesser extent than would occur in the absence of the present method.

This embodiment is particularly useful for preventing passage of any macromolecule containing disulfide bonds

which must be cleaved in order for all or a component of the macromolecule to pass across a cell membrane, particularly a cell surface membrane. In one embodiment in which PDI is inhibited, passage of a toxin, such as diphtheria toxin, across a cell membrane (in this case the endosomal membrane) is totally or partially inhibited and, thus, its cytotoxicity is reduced. Passage of the toxin is reduced by preventing the reductive cleavage of the toxin's disulfide bonds by inhibiting the reductive function of cell membranes, which is capable of cleaving disulfide bonds in membrane-bound macromolecules, including toxins (e.g., bacterial and plant toxins). In a specific embodiment, activation of a toxin, such as diphtheria toxin, is inhibited by inhibiting reductive cleavage catalyzed by the enzyme protein disulfide isomerase. As a result, cytotoxicity of the toxin, such as diphtheria toxin, is less than would be the case in the absence of the present method.

In another embodiment of the present invention in which PDI is inhibited, entry of human immuno-deficiency virus (HIV), or other viruses, particularly retroviruses, containing disulfide bonds in their outer shell, into cells is totally or partially reduced and, thus, the adverse effects of infection are also reduced. Entry of the HIV, and other virus, particularly retroviruses, containing disulfide bond(s) in their outer shell is reduced by inhibiting reductive cleavage of the disulfide bond(s) of the membrane-bound virus at the cell surface. This is effected by inhibiting, directly or indirectly, the reductive function of cell membranes, which Applicant has shown to be catalyzed by PDI.

In a further embodiment of the present invention, the uptake of macromolecules (e.g., hormones, growth factor) present at or delivered to a cell surface receptor via a

carrier to which they are linked by a disulfide bond can be inhibited or enhanced by the present method.

In the case in which uptake of the macromolecule is enhanced, the reductive function of the cell membrane is increased, directly, such as by increasing the amount of surface-associated PDI, and thus increasing the rate at which disulfides of surface-bound effectors are cleaved, increasing the release of effectors (hormones, growth factors) at the cell surface, or indirectly, by altering the configuration or structure of the macromolecule to render the disulfide bonds which must be reductively cleaved for uptake of the macromolecule more readily available to the reductive function of the cell surface membrane.

In either case (i.e., reduced or enhanced uptake of a macromolecule), a preferred embodiment is alteration (reduction or increase) of reductive cleavage at the cell surface of disulfide bonds which link the macromolecule to the carrier. In the present method, reductive cleavage of disulfide bonds in a macromolecule bound to the cell membrane is altered (inhibited or enhanced), directly or indirectly. In the former case, direct alteration of reductive cleavage is effected through the use of agents which act upon the reductive function (e.g., act upon PDI to reduce or increase it activity). Such agents include direct inhibitors of the enzyme PDI, such as those that change the activity of the enzyme (e.g., bacitracin) or block the access of the enzyme to its substrate (anti-enzymes antibodies) and direct enhancers of PDI (e.g. Brefeldin A). In the latter case, indirect alteration is effected through the use of agents which act to render disulfide bonds in the macromolecule more or less accessible to the reductive function (e.g., more or less accessible to PDI). Such agents include those which block the disulfide-containing domains of the macromolecule

ordinarily accessible to PDI (e.g. antibodies against such domains) reducing agents, change the conformation of the macromolecule (e.g., by mutations, mild pretreatment with protease or detergent), and those which change the conditions (e.g., pH) at the cell membrane and, thus, the ability of PDI to catalyze reductive cleavage.

The method of the present invention is useful to prevent adverse effects of toxins, such as diphtheria toxin, on cells. It is also useful in preventing infection (e.g., viral or bacterial) or the spread of an established infection. The method may also be useful in enhancing uptake of macromolecules whose presence within a cell is desired.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graphic representation of the results of three experiments showing that bacitracin protects CHO cells from DT cytotoxicity. Concentration of bacitracin versus percent inhibition of diphtheria toxin cytotoxicity is graphed as a dose dependent curve and results demonstrate protection from the diphtheria toxin by bacitracin. Data from 3 separate, identical experiments were averaged to produce the curve.

Figure 2 is a graphic representation showing in vitro inhibition of PDI-activity by bacitracin and by three membrane-impermeant sulfhydryl blockers. The concentration of each of these inhibitors of PDI-activity versus relative inhibition of PDI is shown and graphed and provides a comparison of the inhibitors' effects. The slope of the bacitracin curve differs from the slopes of the three membrane-impermeant sulfhydryl blockers, which are identical, indicating that the inhibition of PDI by bacitracin works by a different mechanism than that of the three membrane-impermeant sulfhydryl blockers.

Figure 3 is a graphic representation of the effect of anti-PDI antibodies on PDI activity using antibodies directed against either rat or human PDI. This dose dependent curve demonstrates the inhibition of PDI as the antibody dose is increased.

Figure 4 is a graphic representation of bacitracin-induced inhibition of disulfide cleavage of membrane bound ^{125}I-tyn-SS-PDL.

Figure 5 graphically represents the inhibition of HIV infection by DTNB showing the presence of the HIV antigen, p24 (as % of control) in (A) control cells exposed to HIV IIIB for a 2 hour infection period; (B) cells exposed for 30 minutes to 2.5mM DTNB prior to infection and during the 2 hour infection period (n=5); (C) cells infected for 2 hours with virus pretreated with 5.0 mM DTNB for 2.5 hours at 37°C (n=2) and (D) cells infected as in (A) and exposed to 5.0 mM DTNB for 2.5 hours after the infection period(n=2).

Figure 6 is a graphic representation of inhibition of DT cytotoxicity by the anti-PDI antibody, RL77.

Figure 7 graphically represents inhibition of HIV infection by anti-PDI antibodies and bacitracin showing the presence of the HIV antigen, P24, (as % of control) in (A) control cells exposed to HIB IIIB for a 2 hour infection period; (B) cells exposed to monoclonal HP13 antibody (n=4) and (C) cells exposed to 3.0 mM bacitracin (n=5).

Figure 8 is a graphic representation comparing cells treated with Brefeldin A (upper curve) with untreated cells (lower curve).

Figure 9 is a graphic representation demonstrating inhibition of HIV infectivity by Virus treatment with DTT.

Figure 10 is a schematic representation of the amino acid sequence (SEQ ID NO.: 1) of the conserved region of

gp120 (C3 and C4) which contain the binding site of CD4, the V3 loop and the 3 disulfide bonds that are in the proximity of the CD4 binding domains and of the loops they form.

DETAILED DESCRIPTION OF THE INVENTION

An initial, critical step for activation and/or passage of several biologically important macromolecules across an eukaryote cell membrane is cleavage of disulfide bonds present in the macromolecule. Enzymes known to carry out cleavage of disulfide bonds in mammalian cells include protein disulfide isomerase (PDI), thioredoxin and glutaredoxin. As described herein, the critical sulfhydryls which are present at cell membranes, particularly cell surface membranes, and involved in or responsible for the reductive function capable of cleaving disulfide bonds of membrane-bound macromolecules are cysteine residues of PDI. These cysteine residues enable PDI to initiate a disulfide interchange in which the disulfide of the incoming protein is cleaved, thus creating two sulfhydryls on that protein, one of which simultaneously forms a new disulfide with the enzyme, and a new disulfide is formed between that protein and PDI, resulting in a free sulfhydryl on the incoming protein. PDI is generally accepted to be the major cellular catalyst of native disulfide formation and can catalyze net oxidation, reduction or isomerization of disulfide bonds in proteins. While PDI is found in greatest amounts inside the cell, particularly in the endoplasmic reticulum, it has now been shown by Applicant that it is accessible to membrane impermeant inhibitors and thus, must also be present at the cell surface. There is no report that glutaredoxin occurs at the surface of mammalian cells. Comparison of the published sequences shows that glutaredoxin has little homology with PDI, both overall

and at the active site, and is more closely related to thioltransferase than to PDI. Applicants have also demonstrated herein that thioredoxin is not involved in the reductive function of cleaving disulfide bonds of membrane-bound macromolecules at the cell membrane, since it is not inhibited by specific inhibitors of PDI, such as bacitracin, which effectively inhibit the reductive function of the plasma membrane.

As also described herein, it has now been shown that alteration of the catalytic function of PDI results in corresponding alteration in activation of macromolecular toxins which have disulfide bonds, reductive cleavage of which is required for activation and/or penetration of the macromolecule into cells and, thus, for their effects on cells. Specifically, it has been shown that membrane impermeant sulfhydryl blockers cause decreased diphtheria toxin cytotoxicity and decreased viral infection of cells, specifically decreased retroviral infections (HIV) of mammalian cells, and that they do so by blocking the sulfhydryls of PDI, thus abrogating PDI's ability to cleave incoming disulfide via disulfide interchange. PDI activity can be inhibited by agents other than sulfhydryl blockers, such as bacitracin and anti-PDI antibodies, as well as agents which block the disulfide-containing domains of the macromolecule ordinarily accessible to PDI (e.g. antibodies directed against such domains; reducing agents). As shown herein, inhibition of PDI markedly decreases the reductive function of the cell membrane, needed to reduce the disulfide bonds of surface-bound macromolecules, thus reducing the effect of the macromolecules in cells.

Because of the important role of disulfide bonds in maintaining protein structure in general, it is very likely that they play a role in maintaining the structure of viral proteins, and that a mechanism capable of

cleaving disulfide bonds of membrane-bound proteins would also cleave structurally important disulfide bonds of viral proteins interacting with the cell surface.

This invention pertains to a method of altering (i.e., inhibiting or enhancing) reductive cleavage of disulfide bonds in the binding area of specific macromolecular ligands (such as membrane-bound macromolecules or proteins), directly or indirectly, including the naturally occurring disulfide bonds of proteins and peptides, the intermolecular disulfide bonds of naturally assembled proteins and any newly formed or chemically synthesized intra- or intermolecular disulfide bonds. As a result of this methodology, activation of all or a part of macromolecules and/or their passage across a vertebrate cell membrane is altered (inhibited or enhanced).

In one embodiment, activation or passage is eliminated or occurs to a lesser extent in the presence of a direct or indirect inhibitor of the cell membrane reductive function. In particular, inhibition of PDI, directly or indirectly, results in decreased (partial or total) reductive function of cell membranes, and consequently reduced activation or translocation of surface-bound macromolecules, such as toxins, HIV and other viruses, particularly retroviruses, which have disulfide bonds in their outermost proteins.

In one embodiment, activation or passage is elimated or occurs to a lesser extent in the presence of inhibitors of the reductive process that act directly on PDI (e.g., by effects on PDI, such as degrading PDI). Inhibitors of PDI function which inhibit PDI directly include membrane impermeant sulfhydryl blockers (such as 5,5′-dithiobis-(2-nitrobenzoic acid), DTNB; p-chloromercuriphenysulfonic acid, p-CMBS; monobromotrimethylammoniobimane, thiolyte MQ and others); inhibitors of PDI acting by unknown

mechanisms, other than blocking PDI's sulfhydryl groups (e.g., bacitracin), and anti-PDI antibodies, preferably monoclonal antibodies to human PDI.

In another embodiment, activation or passage is eliminated or occurs to a lesser extent in the presence of inhibitors of the reductive process that do not act directly on PDI (e.g., by effects on the membrane-bound macromolecule, such as by blocking disulfide bonds or altering the conformation of the disulfide bond(s)-containing region(s) of a protein, (e.g. a viral protein) rendering the bond(s) less available to the activity of PDI). Such inhibitors include agents or procedures that modify the incoming macromolecule prior to the interaction with the cell surface so as to cleave the disulfide bond(s) of the macromolecule (such as membrane impermeant reducing agents, e.g., dithiothreitol, B-mercaptoethanol GSH), block the disulfide bond(s) of the macromolecule from interaction with membrane-associated PDI (such as antibodies, e.g., monoclonal antibodies directed against a critical disulfide bond-containing domain of a virus) or make disulfide bond(s) of the macromolecule less accessible to membrane-associated PDI, and other agents which inhibit (reduce or prevent) reductive cleavage of disulfide bonds in membrane bound proteins.

In a further embodiment of the present method, reductive cleavage of disulfide bonds which are present in macromolecules and must be cleaved for the macromolecule to pass across a cell membrane and produce its effect on a cell is enhanced, resulting in greater activation of all or a part of the membrane-bound macromolecule than would occur otherwise. Enhancers of reductive cleavage of disulfide bonds of membrane-associated macromolecules can act directly or indirectly on PDI by increasing the catalytic function of PDI (such as by optimizing the presence of cofactors), or by increasing the presence of

PDI at the cell surface (such as by influencing its routing from the endoplasmic reticulum (ER) to the plasma membrane). Enhancers of reductive cleavage can act on the incoming macromolecule by making its disulfide bonds more accessible or more susceptible to surface-associated PDI (such as by biochemically, immunologically, genetically or microenvironmentally induced changes in conformation). For instance, Example 10 and Figure 8 provide evidence that Brefeldin A enhances PDI catalyzed reductive cleavage of disulfide bonds by shifting PDI from the endoplasmic reticulum to the cell surface and as a result, enhances the cleavage of membrane-bound molecules.

The present invention also relates to a method of assaying for agents which inhibit or enhance PDI function, either by direct effect on PDI in an in vitro enzyme assay or by indirect effect on the membrane-bound substrate (e.g., toxin, virus, other disulfide-bond containing model compound). In the present method a compound or molecule (referred to as an agent) to be assessed for its ability to inhibit or enhance PDI function (and, thus, inhibit or enhance activation of a macromolecule referred to as a macromolecule of interest) is made to interact either with the macromolecule of interest or with membrane-associated PDI.

In the present method a compound or molecule (referred to as an agent) to be assessed for its ability to inhibit or enhance PDI function (and, thus, inhibit or enhance activation of a macromolecule, referred to as a macromolecule of interest) is combined with the macromolecule of interest or the recipient cells (e.g., cells in which the macromolecule of interest normally crosses a membrane and has an effect) in an appropriate test system. The resulting assay combination is maintained under conditions appropriate for entry of the macromolecule into recipient cells and its effect on cells it enters; these

conditions are sufficient for reductive cleavage of disulfide bonds in membrane-associated macromolecules. Uptake of the macromolecule in the presence of the agent being assessed is compared with its uptake and effect on recipient cells under the same conditions but in the absence of the agent being assessed (i.e., with control values). Control values can be determined at the time the agent is being assayed or can be previously determined values (standard values determined under control conditions which are the same as those used for assessing the agent, except that the agent is not present). If the effect of the macromolecule of interest is less in the presence of the agent being assessed than in its absence, the agent is an inhibitor of activation of the molecule (presumably through inhibition of reductive cleavage of disulfide bonds in the macromolecule). If the effect of the macromolecule of interest is greater in the presence of the agent being assessed, the agent is an enhancer of activation of the macromolecule (presumably through enhancement of reductive cleavage of disulfide bonds in the macromolecule).

In one embodiment, the assay consists of measuring the cleavage of a model disulfide compound (^{125}I-tyramine-SS-poly(D-lysine)), as a way of measuring a biologic function associated with disulfide cleavage (diphtheria toxin cytotoxicity, HIV infection of H9 cells) in the presence or absence of the agent, to assess whether the agent is capable of inhibiting (or enhancing) the reductive process. If the measured cleavage of the biologic functions is less in the presence of the agent being assessed than in its absence, the agent is an inhibitor of the membrane-associated reductive process. If the measured cleavage or functions is greater in the presence of the agent being assessed, the agent is an

enhancer of the membrane-associates reductive process. Agents assayed can be existing compounds or molecules, such as those in chemical libraries or available through commercial sources, or can be compounds or molecules designed to inhibit or enhance reductive cleavage of disulfide bonds in membrane-associated macromolecules by inhibiting or enhancing PDI function.

The present invention further relates to molecules, particularly those identified by the assay method described above and in the examples, which act as inhibitors or enhancers of activation of macromolecules by inhibiting or enhancing reductive cleavage of disulfide bonds.

The oxidoreductive activity of membrane-associated PDI serves many important functions with different biological endpoints, and therefore the inhibition or enhancement of membrane-associated PDI activity has many areas of application. Examples of such applications are:

a) The released functional moiety of a heterodimeric protein has biological effects independent of its uptake or translocation, such as a "signal transduction effect" or an enzymatic effect. The first instance is illustrated by the postulated reductive cleavage of insulin following insulin-receptor interaction. The second instance is illustrated by the reductive cleavage of tetanus toxin which releases an activated light chain endowed with endopeptidase activity.

b) The functionally significant result of PDI-mediated reductive cleavage is the formation of a new disulfide bond between PDI and part of the incoming ligand. Such a possibility has been postulated for insulin and other growth factors. It would lead to the disulfide bonding of an activated moiety of a hormone to its receptor. Significant and biologically relevant conformational changes can be expected to occur in polypeptides released

from a disulfide bond as well as in receptor proteins following their disulfide bonding to an incoming polypeptide.

c) The cleavage of a disulfide bond in the binding domain of a ligand will cause conformational changes associated with biologic effects other than those mentioned above. The release of $Fe^{2}+$ from transferrin is mentioned here to illustrate this possibility. After binding to its specific surface receptor and internalization by receptor-mediated endocytosis, this iron transporting protein reaches an early endosome where it releases its iron content. The mechanism of this release is not yet elucidated. Since transferrin is known to contain numerous disulfide bonds, we postulate that PDI-mediated cleavage of one or more such bonds might cause conformational changes that critically contribute to iron release.

d) Disulfide-bonded, membrane-associated PDI (PDI-SS-X) acts as an oxidase and catalyzes the formation of a new disulfide bond with any extracellular protein bearing a free thiol (Y-SH). PDI would in this case catalyze a thiol:disulfide exchange of the type:

Y-SH + PDI-SS-X → Y-SS-X+PDI-SH or

Y-SH + PDI-SS-X → Y-SS-PDI + SH-X

Both reactions can be associated with a number of important biologic effects, such as: a) The activation of X-SH (enzyme activity, critical conformational change, critical binding to other macromolecules); b) the activation of X through disulfide bonding to a partner other than PDI; c) activation of Y through disulfide bonding to either PDI or X.

e) A cascade of thiol:disulfide interchanges may be initiated by the bonding of an external soluble protein (SH-Y) with membrane-associated PDI (Y-SS-PDI), leading to

the subsequent disulfide bonding of PDI with a membrane protein SH-P'(Y-SH + PDI-SS-P'), followed by the formation of a new disulfide bond between P' and P", another membrane protein, (PDI-SH + P'-SS-P") catalyzed by the oxidative function of PDI. The end result is the dimerization of P' to P" through a disulfide bond. It has been proposed that receptor proteins of the PDGF receptor undergo such a dimerization upon ligand receptor interaction.

f) The prime function of surface-associated PDI is to create a disulfide bond between two cells. All this would require is that cell A would provide a free thiol and cell B would provide an oxidized PDI (PDI-SS-X), where X is a membrane protein with a cysteine residue on cell B. PDI would act in this case as an oxidase catalyzing the formation of a new disulfide bond between A-SH of cell A and PDI-SS-X of cell B, resulting in A-SS-X-B + SH-PDI. Such a covalent cell-cell interaction could similarly result from the cleavage of an exposed disulfide on cell A (Y-SS-W) and an exposed SH-PDI on Cell B leading to Y-SS-PDI or W-SS-PDI. Such thiol:disulfide interchanges could be associated with biologically important cell-cell interactions, such as contact inhibition of normal cells, aggregation of platelets or other blood cell, adhesion of platelets or other blood cells to endothelial cells, adhesion of aging erthyrocytes to spleen cells.

The following is a description of work which demonstrated the critical role of cell surface sulfhydryls in reductive cleavage of disulfide bonds in membrane-bound macromolecules, specifically proteinaceous macromolecules; the inhibition of reductive cleavage of a model disulfide conjugate at the surface of CHO cells in the presence of membrane impermeant sulfhydryl inhibitors; and the relationship of these findings to the decreased cytotoxicity of toxins, as exemplified by diphtheria

toxin, and the decreased infection of a virus, specifically HIV. Decreased cytotoxicity of the toxin and decreased infectivity of HIV virus are both related to decreased cleavage of critical disulfide bonds by the reductive function of cell surface membranes that normally occurs upon attachment of the toxin or the virus to its cell surface receptors. Also described is identification of the reductive function of cell surface membranes as protein disulfide isomerase (PDI) and application of this finding to altering PDI function (activity and/or levels) at cell membranes, both cell surface membranes and intercellular membranes (e.g., in the endoplasmic reticulum and the Golgi apparatus).

Initially, cleavage of the disulfide contained in a membrane-bound model compound was shown to be inhibited through the use of membrane impermeant inhibitors of surface sulfhydryls. These inhibitors prevent cleavage of disulfide bonds of that model compound at the plasma membrane. A radioiodinated tyramine, ([^{125}I]tyn) derivatised with N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) was reacted with an undegradable carrier, 3-thiopropionyl poly(D-lysine) (PDL-SH) to produce the model disulfide compound [^{125}I]tyn-SS-PDL. Cleavage of the disulfide bond (SS) results in the release of 3-thiopropionyl[^{125}I] tyramine ([^{125}I]tyn-SH) which is easily measured as released trichloroacetic acid-soluble radioactivity. This model compound provides a rapid, sensitive, and direct assay for detection of disulfide cleavage occurring at a cell membrane, since it is not degraded by cellular enzymes that catalyze breakdown of proteins and is stable at acidic pH, which is characteristic of endocytic vesicles. As described in Example 1, model compound [^{125}I]tyn-SS-PDL was bound to the surface of Chinese hamster ovary (CHO) cells.

Simultaneously, these CHO cells were exposed to the membrane-impermeant sulfhydryl inhibitor 5,5'-dithiobis-(2-nitrobenzioc acid) (DTNB) or p-chloromercuriphenylsulfonic acid (pCMBS). Each inhibitor caused a marked inhibition of disulfide cleavage during the first 30 min of pulse chase, following labeling of the cell surface with the conjugate at 0°C.

During the initial 15 minutes of chase at 37°C the surface sulfhydryl inhibitors inhibited disulfide bond cleavage of the model compound by more than 90%, indicating surface sulfhydryl groups are involved in cleaving disulfides in the earliest period of chase. Supporting this conclusion was data from the same experiment conducted on a model compound lacking a disulfide bond. See also Feener, E.P., et al., JBC, 365:18780-18785 (1990).

Subsequently, cytotoxicity of diphtheria toxin containing a critical disulfide bond has been shown to be prevented by the same membrane impermeant inhibitors of surface sulfhydryls. The diphtheria toxin, produced by Corynebacterium diphtheriae, kills cells by irreversibly inhibiting protein synthesis of exposed cells. This toxin is secreted as a single chain precursor comprised of two polypeptide chains, A and B, linked by a disulfide bond. It is generally accepted that both chains of the diphtheria toxin enter cells by receptor-mediated endocytosis, in which a specific receptor protein on the plasma membrane surface recognizes the B chain of the diphtheria toxin, and firmly binds it. The segment of the plasma membrane containing the protein-receptor-diphtheria toxin complex then invaginates, forming an endosome, which is a new intracellular membrane-bound vesicle completely surrounded by cell cytoplasm. The endosome then undergoes acidification to around pH 5.0. The B-chain of the nicked diphtheria toxin undergoes

conformational changes due to the acidic environment of the endosome. As a result, the B-chain inserts itself into the endosomal membrane which facilitates the passage of the A-chain into the cell's cytoplasm where irreversible inhibition of protein synthesis occurs. Clearly this translocation of chain A must be preceded by a separation of chains A and B, i.e., by the cleavage of the interchain disulfide bond.

As described in Example 2, that cleavage of the interchain disulfide bond of the diphtheria toxin requires free sulfhydryl groups at the cell surface whether or not the cleavage occurs before, during or after formation of the primary endocytic vesicle. Upon vesiculation, these surface sulfhydryls become situated at the inner surface of the newly formed endosomes, during the initial step of receptor-mediated endocytosis. Upon addition of membrane-impermeant sulfhydryl blockers, the cytotoxicity of the diphtheria toxin was markedly inhibited. Two sulfhydryl blockers used for that purpose were 5, 5'-dithiobis(2-nitrobenzoic acid) (DTNB), and p-chloromercuriphenyl-sulfonic acid (pCMBS)). They were present in the buffer used to wash the cell monolayer prior to addition of the toxin, as well as in the toxin-containing medium.

Control experiments related to Example 2 demonstrated that inhibition of the diphtheria toxin cytotoxicity was due to inhibition of disulfide bond cleavage of the macromolecule and not due to inhibitory effects on 1) diphtheria toxin activity prior to binding to diphtheria toxin receptors, 2) the receptor-mediated endocytosis process itself, or 3) the endosome acidification process. The data presented below supports the conclusion that disulfide bond cleavage, necessary for entry of several macromolecules into a cell, requires a sulfhydryl-containing enzyme (PDI) originally present at the cell surface and that, through receptor-mediated endocytosis,

the enzyme becomes situated at the inner surface of newly-formed endosomes. Therefore, impermeant inhibitors of disulfide bond cleavage present in the medium become part of the fluid volume of the endosomes and remain membrane-impermeant in newly-formed endosomes. In this way, these membrane-impermeant inhibitors can inhibit entry of macromolecules, such as the cytotoxic chain of diphtheria toxin (Chain A), which require passage through endosomal membrane and which require cleavage of a critical interchain disulfide bond as an initial step for its translocation across the endosomal membrane. See also, Ryser, H.J-P. et al., JBC, 266:18439-18442 (1991).

Having been able to link the membrane-associated mechanism of disulfide cleavage with the activation of diphtheria toxin, it appeared compelling to look for other biological functions that might be served by this newly discovered reductive function of the plasma membrane and might be inhibited by the same sulfhydryl blockers or other inhibitors of PDI.

Disulfide bonds play a central role in the generation and maintenance of the 3-dimensional conformation of proteins such as those found in the outer lattice of many viruses. It was anticipated, therefore, that cleavage of intramolecular disulfides in other functional membrane-bound proteins, particularly the envelope proteins of membranebound viruses, might be associated with important biological functions.

Because of the great medical significance of the worldwide HIV epidemic, the possibility was investigated that the cleavage of critical disulfide bonds in the outer proteins (envelope proteins) of HIV might play a role in HIV infection and that inhibiting the cleavage of such bonds with DTNB or other membrane impermeant sulfhydryl blockers might inhibit HIV infection of human cells. The gp120 glycoprotein of HIV I specifically interacts with

CD4, the virus receptor on the surface of human lymphoid cells. This protein is known to contain 9 disulfide bonds, two of which are situated in the binding domain of gp120. See Figure 10. It was postulated that the interaction of HIV with its receptor brings the binding domain of gp120 in close contact with the membrane-associated reductive cleavage mechanism on human cells, an interaction that can effect the cleavage of one or more disulfide bonds of gp120. Since cleavage of protein disulfide bonds is known in general to cause major conformational changes, cleavage of specific disulfides of gp120 can be expected to cause conformational changes leading to and required for virus penetration and infection of human cells. DTNB concentrations used to inhibit diphtheria toxin toxicity on CHO cells were used to test whether they similarly inhibited HIV infection of human lymphoid cells. These experiments are described in Example 3 and show that the sulfhydryl inhibitors inhibited HIV infection of human lymphoid cells.

Reductive cleavage of disulfide bonds commonly occurs as a result of a thiol:disulfide exchange reaction, either in the presence of excess thiols or in the presence of an enzyme with oxidoreductase activity. Since the two experimental systems in which cleavage of protein disulfide bonds was demonstrated do not contain an excess of thiols, it was concluded that the cleavage was enzymatically driven. Three enzymes are known to catalyze such reductions, namely PDI, thioredoxin and glutaredoxin. The possible role of PDI was tested first, because of the availability of known and powerful PDI inhibitors.

PDI is a well characterized multi-functional enzyme which plays a critical role in the formation of disulfide bonds in nascent proteins. Consistent with this role, the major subcellular localization of PDI is the endoplasmic reticulum, the first compartment reached by newly

synthesized proteins. There have been controversial findings regarding the possibility that PDI might be present also at the cell surface. For instance, Varandani presented evidence that the insulin disulfide transhydrogenase (another appellation for PDI) was detectable at the surface of pancreatic cells. Varandani, P.T., et al., Biochem. Biophys. Acta, 538:343-353 (1978), Kaetzel, however, found no evidence that PDI was present at the surface of human placenta and rat liver cells. (Kaetzel, C. S. et al., Biochem. J., 241:39-47 (1987). Attention to the possible surface localization of PDI was diverted by the prominent role PDI plays in the ER and by the fact that the carboxyl-terminal sequence, the KDEL retention signal, is credited for effectively retaining it in the ER. The applicant's data, however, indicate that this retention is incomplete and that enzymatically active PDI is transported from the ER to the plasma membrane. This view is supported by the finding that the binding protein for the hormone triiodothyronine, which is known to be identical to PDI, has also been identified at the cell surface, and by the finding that PDI has been detected at the cell surface by an immunoelectron-microscopic probe recognizing the KDEL sequence. Yoshimori, et al., J. of Biol. Chem. 265:15984-15990 (1990).

In order to obtain evidence for the participation of PDI in the reductive function at the surface of CHO cells, several strategies were used. First it was ascertained that the non-specific inhibitors of PDI (DTNB, pCMBS, thiolyte MQ), and the specific PDI inhibitor bacitracin (Example 6, Fig. 2) as well as anti-PDI monoclonal antibodies (Example 6, Fig. 3) caused dose-dependent inhibitions of PDI activity in vitro. Second, it was shown that DT cytotoxicity was inhibited by bacitracin (Example 5 and Fig. 1) and by monoclonal anti-PDI-

antibodies (Example 8, and Fig. 6). Third, it was shown that the cleavage of membrane-bound ^{125}I-tyn-SS-PDL was inhibited by bacitracin (Example 4 and Fig. 4) and by monoclonal anti-PDI antibodies (Example 7). These examples and figures clearly demonstrate that PDI is involved in two distinct processes of membrane-associated reductive cleavage, namely the reductive activation of membrane-bound DT and the release of ^{125}I-tyn-SH from membrane-bound ^{125}I-tyn-SS-PDL.

The similarity between the inhibitory effects of bacitracin and sulfhydryl reagents suggests that the critical surface sulfhydryls blocked by DTNB and pCMBS and required for the thiol-disulfide interchange are the cysteine sulfhydryls present at the catalytic sites of PDI. Unlike DTNB, bacitracin does not contain functional groups that are known to block free sulfhydryls and is likely, therefore, to act by a different, albeit unknown, mechanism. This view is consistent with the fact that the slope of its dose-inhibition curve differs from that of three sulfhydryl reagents (Fig. 2), and with the observation that it had an additive effect in suppressing DT cytotoxicity when given together with sulfhydryl blockers. Since ^{125}I-tyn-SS-PDL and DT interact with the plasma membrane at different sites, it can be inferred that PDI is present both in domains that interact non-specifically with the positive charges of poly(D-lysine) (PDL) and in the receptor area that specifically binds the B chain of DT. The data of Fig. 4 indicate that bacitracin can act before internalization of ^{125}I-tyn-SS-PDL.

It should be noted that monoclonal antibodies directed against rat or human PDI inhibited the activity of calf liver PDI <u>in vitro</u> at the surface of CHO cells. This crossreactivity is consistent with the extensive

homology of human, bovine and rat PDI (Parkkonen, T., et al., Biochem. J., 256:1005-1011 (1988)). Crossreactivity between human, bovine and rat PDI was noted with polyclonal antibodies against human and rat PDI (Kaetzel, C.S., et al., Biochem. J., 241:39-47 (1987)), as well as with a monoclonal antibody to purified triiodothyronine binding protein (T3BP) (Flynn, D.C., et al. J. Virol., 64:3643-3654 (1990)) now known to be identical to PDI (Freedman, R.B., Cell, 57:1069-1072 (1989)). See Example 6 and Fig. 3. Using the methods and reagents described herein, the ability of bacitracin and antibodies directed against PDI, were also assessed for their ability to inhibit HIV. If anti-PDI antibodies bind to PDI and reduce or abolish its ability to reductively cleave disulfide bonds at the cell surface membrane, PDI will be unable to cleave the critical disulfide bonds present in the gp120 glycoprotein of HIV, a cleavage that is required to initiate virus penetration into the cells. As demonstrated in Example 9, the same reductive process plays a role in the penetration of membrane-bound HIV and HIV infection of H9 cells is markedly reduced by anti-PDI antibodies and bacitracin, an inhibitor of PDI. These results, in conjunction with the inhibition of HIV infection by DTNB in Example 3, imply that HIV and its target cell engage in a thiol:disulfide interchange mediated by PDI and that the reduction of critical disulfides in viral envelope proteins is an initial event that triggers conformational changes required for HIV penetration and cell infection. This is reasonable to expect since three different types of PDI inhibitors (DTNB, anti-PDI antibodies and bacitracin) having no chemical or biochemical characteristics in common, except their inhibition of PDI in vitro, inhibit HIV infection. These findings identify novel targets for HIV-therapy.

The strategies of using bacitracin and anti-PDI-antibodies to block reductive function of the plasma membrane of CHO cells were both successful and provide strong evidence that the three different reductive processes now shown to occur at the cell surface (namely, the cleavage of membrane-bound ^{125}I-tyn-SS-PDL, the cytotoxicity of diphtheria toxin and the conformational changes in gp120 required for HIV infection) are driven by the enzyme PDI.

Based on this discovery, similar inhibitions of viral infection can be obtained by blocking or cleaving the viral disulfides prior to virus-cell interaction, thus preventing the viral disulfides from interacting with PDI and preempting the viral infection process. As described in Example 11, HIV alone was treated with the reducing agent dithiothreitol (DTT) prior to virus-cell interaction. This inactivated the virus in a dose-dependent fashion up to a 100% inhibition or infectivity. See Figure 9. Viral infections can also be inhibited with antibodies directed against the discrete gp120 domains that contain the critical disulfide bonds which are illustrated in Figure 10. Of the nine disulfide bonds of gp120, two are situated in the binding domain that interacts with CD4 receptor, and one disulfide is in an adjacent fusogenic domain. Since the sequence of gp120 is known, it is reasonable to expect one skilled in the art could either excise these domains or synthesize the corresponding disulfide-containing sequences and use these peptides to raise antibodies against them. According to the 1991 annotated sequence of gp120 from isolate BH10 (NCB1, Natl. Library of Med. NIH), the two disulfide bonds closest to the CD4 binding site are between cysteines 378-445 and cysteines 385-418. CYS378 is close to a sequence in C3 (364-370) believed to participate in CD4 binding.

Larsen, C. et al., Membrane Interactions of HIV, eds. Alola, R.C. et al. (Wiley-Liss, Inc., New York, 1992) 143-146. CYS418 is at the start of the primary CD4 binding site in C4 (418-437). Larsen, C. et al., Membrane Interactions of HIV, eds. Alola, R.C., et al., (Wiley-Liss, Inc., New York, 1992) 143-146. The primary CD4 binding site is thus framed by two disulfide bonds which are plausible targets for a PDI-mediated interchange reactions. A third disulfide bond (CYS296-331) forms the V3 loop, which is believed to have fusogenic properties. Larsen, C. et al., Membrane Interactions of HIV, eds. Alola, R.C. et al., (Wiley-Liss, Inc., New York, 1992) 143-146. The base of the V_3 loop where this disulfide is located, although distant in the sequence by some 50 to 100 amino acids from important elements of the CD4 binding site, is believed to be situated in close proximity of the binding site in the tertiary structure of the native gp120. Moore, J. P. et al., J. Virol. 67:4785-4796 (1993). The variable regions V3 and V4 are thought to include amino acids 297-328, and 394-413, respectively. The conserved region C3 and C4 follow V3 and V4, repectively. Recent reviews and articles on the CD4-gp120 interaction in AIDS refer to the work of Leonard C.K. et al., Biol. Chem., 265: 10373-10382 (1990), but do not comment on the possible biologic significance of the 9 disulfide bonds of gp120. (Larsen, C. et al., Membrane Interactions of HIV, eds; Capon, D.J. et al., Annu. Rev. Immunol., 9: 649-678 (1991); Moore, J. P. et al., J. Virol. 67:4785-4796 (1993)). This may be because it had been assumed that the cysteines involved were buried in the native HIV glycoprotein, but more likely because the reductive function of the plasma membrane was not yet known. The advantages of this new approach which would

block the viral disulfides rather than the cellular enzyme are substantial.

The method of administering an effective dose of inhibitors of disulfide bond cleavage in vivo is by any means presently available to one skilled in the art.

Thus, as described herein, it has now been shown that the reductive function of cell membranes, and particularly the cell surface membrane, can be effectively altered. In addition, strong evidence is provided that the reductive function of the eukaryotic cell surface membrane is catalyzed by PDI and that inhibitors which are sulfhydryl blockers of PDI-specific inhibitors are effective in inhibiting PDI activity and the effects on cells of membrane-bound macromolecules which contain disulfide bonds which must be cleaved for the macromolecules to cross the membrane and exert their respective effects on their target cells.

In particular, it has now been shown that the cytotoxicity of a toxin which contains disulfide bonds which must be cleaved for the toxin to enter cells and exert cytotoxic effect is reduced by blocking the reductive function of the eukaryotic cell membrane. Inhibition of the cytotoxic effect of the toxin, specifically diphtheria toxin, results from the use of sulfhydryl blockers and from the use of a PDI-specific inhibitor. That is, DTNB (a non-specific sulfhydryl blocker) and bacitracin (a specific PDI inhibitor) have been shown to reduce diphtheria toxin cytotoxicity in eukaryotic, including mammalian, cells.

As a result of the work described herein, the cytotoxicity of other toxins which must undergo reductive cleavage or processing at a cell membrane, particularly the cell surface membrane, and the membrane of nascent endosomes, can be inhibited. This can be done by contacting cells in which cytotoxicity is to be inhibited

(totally or partially) with a sulfhydryl blocker or a PDI-specific inhibitor. Sulfhydryl blockers include, but are not limited to, DTNB, pCMBS and thiolyte MQ. PDI-specific inhibitors include, but are not limited to, bacitracin and anti-PDI antibodies, which can be polyclonal or monoclonal. A sulfhydryl blocker or a PDI-specific inhibitor can be administered to (contacted with) cells individually or in various combinations (e.g., combinations of two or more sulfhydryl blockers; two or more PDI-specific inhibitors; sulfhydryl blocker(s) and PDI-specific inhibitor(s)).

The work described in Example 2 and Example 5 shows the effectiveness of the present method in inhibiting the cytotoxicity of diphtheria toxin by an inhibitory effect at the cell surface membrane. It is reasonable to expect other toxins (e.g., pseudomonas toxin E, gelonin, toxic ribonucleases, modeccin pseudomonas endotoxin, choleratoxin, tetanus toxin) can be similarly inhibited by inhibiting the reductive function of the cell surface membrane. Alternatively, although the cytotoxic effects of ricin were not inhibited by the same cell membrane impermeant inhibitors (See Ryser, H. J.-P. et al., J. Biol. Chem., 266:18439-18442(1991)), it is reasonable to expect that inhibition of reductive cleavage by PDI at an intracellular membrane will inhibit cytotoxicity of ricin and toxins which are processed by the cell in a similar manner. Diphtheria toxin and ricin differ markedly in their intracellular paths and sites of entry in the cytoplasm. Unlike diphtheria toxin, ricin is translocated from late endocytic structures, presumably from elements of the Golgi structure. It is likely, therefore, that the reductive cleavage critical for translocation also occurs at different sites, which may explain why inhibition of surface sulfhydryls by membrane-impermeant fails to inhibit ricin cytotoxicity. It is reasonable to conclude

that both diphtheria toxin and ricin reach their respective translocation sites in a membrane-bound state and that in both cases, cleavage requires the presence of membrane-bound sulfhydryls. It is also reasonable to suggest that membrane-permeant sulfhydryl blockers (such as NEM or N-ethyl maleimide, a membrane permeant sulfhydryl blocker and a known inhibitor of ricin cytotoxicity) or PDI-specific inhibitors which act at an internal cell membrane will be effective in reducing ricin cytotoxicity and cytotoxicity of other toxins processed in a similar manner by eukaryotic cells.

The surface associated reductive mechanism initially revealed by the cleavage of ^{125}I-tyn-SS-PDL may serve functions other than the reductive activation of DT. For instance, alpha 2-macroglobulin, a well characterized macromolecular carrier that interacts with a specific surface receptor, has been shown to form disulfide bonds with platelet derived growth factor (Huang, J.S., et al., Proc. Natl. Acad. Sci. USA, 8:342-346 (1988), interleukin-1-beta (Borth, W., et al., J. Immunol., 145:3747-3754 (1990)) and the deglycosylated A chain of ricin and ricin immunotoxins (Ghetie, M-A., et al., Cancer Res., 51:1482-1487 (1991)). A reductive mechanism capable of cleaving the interchain of membrane bound DT and the intrachain disulfide of ^{125}I-tyn-SS-PDL might be expected to cleave the disulfide linkage of these membrane-bound alpha 2-macroglobulin conjugates and release the carried functional proteins at the cell surface or in nascent endosomes. Other recent data have indicated that disulfides present in viral envelope proteins of viruses other than HIV may be cleaved upon virus-cell interaction (Myer, W.J., et al., J. Virol., 66:3504-3513 (1992)) and that inhibition of this reductive cleavage may interfere with virus infection (Brown, D.T., et al., Semin. Virol.,

3:519-527 (1992)). These new perspectives broaden the possible role of membrane-associated PDI and the spectrum of action of an enzyme already known for its multifunctional properties.

In the method of the present invention of reducing or preventing cytotoxicity of a toxin, cells in which cytotoxicity is to be reduced or prevented are contacted with a sulfhydryl blocker, a PDI-specific inhibitor or both in an amount sufficient to inhibit (totally or partially) reductive cleavage of the toxin macromolecule. In the case of diphtheria toxin and similar cell-surface-bound toxins, the sulfhydryl blocker(s), used will preferably be membrane-impermeant. In the case of ricin and similarly processed toxins, the sulfhydryl blocker(s) used will preferably be membrane permeant. The sulfhydryl blocker(s), PDI-specific inhibitor(s) (e.g., bacitracin, anti-PDI antibodies) or a combination thereof (referred to as drug(s)) can be administered to an animal, particularly a mammal, including humans, using known methods. The amount of each necessary to reduce cytotoxicity of the toxin will be determined empirically, taking into consideration, for example, the particular toxin, the drug (e.g., sulfhydryl blocker(s), bacitracin, anti-PDI antibodies being administered), the drug's toxicity, the size and age of the individual and the severity of the condition being treated or prevented. The drugs can be obtained from commercial sources or can be produced using known methods. The drug(s) can be included in a formulation which is administered to an individual being treated; such a formulation can also include a physiologically compatible carrier (e.g., a physiological buffer), stabilizers, flavorants, adjuvants and other components. The drug(s) can be administered by a variety of routes (e.g., topically, parenterally, intravenously,

intraperitoneally), and the components of the formulation will be selected accordingly.

It has also been shown that the infectivity of a virus, particularly a retrovirus such as the human immunodeficiency virus or HIV, is inhibited by blocking the reductive function of the cell surface membrane. Membrane impermeant sulfhydryl blockers, bacitracin and anti-PDI antibodies inhibited HIV infection of mammalian (human) cells. In a manner similar to that described above for toxins, infectivity of HIV and other viruses, particularly retroviruses, can be inhibited by the present method. In the present method, cells to be protected against HIV (or other viral) infection are contacted with drug or drugs, which are sulfhydryl blocker(s), a PDI-specific inhibitor(s) or both in sufficient quantity and by an appropriate route to result in reduced infection of the cells by the HIV or other virus (i.e., infection is totally inhibited or occurs to a lesser extent than occurs in the absence of the present method). In addition, it has also been shown that infectivity of a virus containing critical disulfides bond domains in the outer proteins, particulary HIV, is inhibited with reducing agents. It is reasonable to expect that infectivity of other viruses which contain critical disulfide bond domains in their outer proteins (e.g., hepatitis virus, herpes virus, papilloma virus, enterovirus or paramyxovirus) can be similarly inhibited by membrane impermeant reducing agents or monoclonal antibodies directed against the disulfide bond-containing domains. The drug(s) administered can be in a formulation suitable for the route of administration used (topical, parenteral, intravenous, intraperitoneal). The amount to be administered and the frequency of administration can be determined empirically and will take into consideration the age and size of the person being treated, the stage of infection by HIV or other virus

(e.g., prior to infection, soon after infection occurs or in later stages of infection) and the particular virus.

Alternatively, the uptake of macromolecules (e.g., growth hormone, interleukines, lymphokines, hematopoietic stimulating factors, specific epithelial, endothelial and fibroblast stimulating factors, transforming growth factors) present at or delivered to a cell surface receptor via a carrier to which it is linked by a disulfide bond can be inhibited or enhanced by the present method.

The present invention is illustrated by the following Examples, which are not intended to be limiting in any way.

EXAMPLE 1 Inhibition of Disulfide Cleavage in a Strongly Membrane-Bound Model Compound Containing an Intramolecular Disulfide Bond by Membrane Impermeant Sulfhydryl Inhibitors

The following materials and methods used were: pCMBS, DTNB, N-ethylmalamide (NEM), poly(D-lysine), 60 kDa, (PDL,) were purchased from Sigma. [^{125}I]PDL was prepared by iodination of Bolton-Hunter reagent-modified PDL, as described. [^{125}I]-tyramine-SS-poly(D-Lysine) ([^{125}I]tyn-SS-PDL) was prepared by disulfide exchange of 3-thiopropionyl PDL with 3-(2-pyridyldithio)propionyl [^{125}I]tyramine, i.e. with SPDP-[^{125}I}-tyramine.

Exposure of [^{125}I]tyn-SS-PDL to reducing agents in vitro releases trichloroacetic acid-soluble 3-thiopropionyl-[^{125}I]iodotyramine ([^{125}I]-tyn-SH). Continuous exposure of cultured CHO monolayers to 1 μg/ml [^{125}I]tyn-SS-PDL resulted in a time-dependent release of trichloroacetic acid-soluble radioactivity over a period of 6 h. The acid-soluble radioactive compound released had the same mobility on thin-layer chromatography as that

resulting from GSH-mediated reduction of the probe in vitro. No acid-soluble radioactivity was released when cells were exposed to ^{125}I-poly(D-lysine), a non-degradable control. A small amount of acid-soluble radioactivity was released when [^{125}I]tyn-SS-PDL was incubated in cell-free medium at 37°C (Fig. 1). To eliminate this unwanted background, cells were prelabeled at 0°C and reincubated in label-free medium at 37°C, a procedure used in subsequent experiments. Using ^{125}I-PDL, we showed that detachment of pre-bound conjugate from the cell surface was only 3% of the total cell-bound radioactivity after 1 h at 37°C. The release of trichloroacetic acid-soluble radioactivity was totally inhibited when NEM (10mM) was added to the chase medium, indicating that the reduction of [^{125}I]tyn-SS-PDL requires cell-associated sulfhydryl groups.

The kinetics of disulfide cleavage of [^{125}I]tyn-SS-PDL was compared with the kinetics of proteolysis of ^{125}I-PLL. Proteolytic degradation was preceded by a lag of approximately 30 min. This lag corresponds to the time required for surface-bound ^{125}I-PLL to reach lysosomes, as determined by subcellular fractionation of pulse-labeled cells. In contrast, the maximal rate of cleavage of [^{125}I]tyn-SS-PDL occurred without lag. This suggested that, unlike proteolysis, disulfide cleavage begins at the cell surface and/or in early endosomes. Exposure of monolayers at low temperature could not be used to distinguish between these two possibilities since low temperature, in addition to inhibiting endocytosis, also strongly inhibited disulfide cleavage of [^{125}I]tyn-SS-PDL in vitro in the presence of reducing agents. As an alternative approach, two membrane-impermeant sulfhydryl reagents, DTNB and pCMBS, were used to inhibit cell surface sulfhydryl groups that might be involved in a

disulfide interchange reaction, resulting in the disulfide cleavage of membrane-bound macromolecules. These reagents caused a marked and concentration-dependent inhibition of disulfide cleavage during the first 30 min of chase and had only a small effect on the proteolytic breakdown of ^{125}I-poly-(L-Lysine) (^{125}I-PLL). Inhibition was thus not due to decreased endocytosis since the treated cells were able to internalize ^{125}I-PLL normally prior to its proteolytic degradation. Internalization of PLL is very rapid and efficient. The inhibitory effect of pCMBS was therefore analyzed as a function of time by adding the inhibitor for a limited period, beginning at the earliest chase times. During the first 5 or 15 min of chase pCMBS inhibited the release of [^{125}I]iodotryamine >90%. When the inhibitor was added after 5 min of chase, no reduction was detected during the subsequent 10 min. When added for 30 min after 30 or 60 min of chase, pCMBS's inhibitory effect dropped to 38 and 6.4% of the total reduction, respectively. The data indicate that surface sulfhydryl groups are involved in the earliest phase of reduction of cell-associated [^{125}I]tyn-SS-PDL. The fact that reduction was measured between 5 and 15 min when pCMBS was added after 5 min of chase suggests that reduction does not start upon formation of the initial endocytic vesicles (early endosomes). The fact that the efficiency of inhibition decreased between 15 and 30 min of chase and dropped to 6.4% of total cleavage between 60 and 90 min indicates that a second process of reduction sets in between 15 and 30 min which becomes predominant at later times. Between 30 and 120 min of chase, reductive cleavage of the conjugate proceeds almost at the same rate in the presence and absence of inhibitor and must therefore occur at sites that are not accessible to the membrane-impermeant inhibitor.

EXAMPLE 2 Inhibition of the Diphtheria Toxin Cytotoxicity (DT) by Addition of DTNB and pCMBS, Membrane-Impermeant Inhibitors of Disulfide Bond Cleavage.

Additional materials and methods used were: dithiothreitol (DTT), ricin. DT was from List Biological Laboratories, Inc., Campbell, CA. Tissue culture products were from GIBCO. Tissue culture flasks and microwell plates were from Falcon, Oxnard, CA. CHO cells were from the American Tissue Culture Collection.

Inhibition of Protein Synthesis by Toxins. CHO cells were grown to confluence in 35mm dishes in α-minimal essential medium supplemented with fetal bovine serum. They were briefly washed with prewarmed serum-free Eagle's medium and incubated for 2 h at 37°C in serum-free Eagle's medium containing DT or ricin. For cells exposed to DTNB and pCMBS, these inhibitors were present both in the preliminary wash and in the toxin-containing medium. After a wash with Earle's balanced salt solution, the cells were reincubated for 90 min at 37°C in serum-free Eagle's medium low in unlabeled amino acids (1:20) containing a mixture of ^{14}C-labeled amino acids (0.4 μC_i/ml). The cytoxicity of DT was assessed by measuring its inhibitory effect on the amino acid incorporation into cellular proteins. Label incorporation into newly synthesized proteins was measured.

Cellular Uptake of ^{35}S-Labeled Lysosomal Enzymes. Uptake of lysosomal enzymes via the mannose S-phosphate (Man-6-P) receptor was measured using NH_4Cl-induced secretions of CHO cells grown in the presence of [^{35}S]methionine. Confluent cultures grown in 35mm dishes were exposed to medium containing $2x10^5$ cpm/dish.

Isolation of Early Endosomes. Confluent cell monolayers in 175 cm flasks were washed with serum-free α-minimal essential medium and pulse-labeled for 15 min at

37°C with 1 μg/ml [^{125}I]PLL. The cells were then harvested with a brief trypsinization at 4°C and washed with Earle's balanced salt solution. The cells were resuspended in 0.25M sucrose containing 1mM EDTA and 10mM HEPES, pH 7.4, and homogenized using a ball-bearing homogenizer to approximately 90% breakage as determined by trypan blue exclusion. The postnuclear supernatant from a centrifugation at 800 x g for 10 min was layered on a 17% Percoll gradient in 0.25 M sucrose and centrifuged for 1 h at 34,500 x g in a Sorvall SV288 vertical head rotor. Fractions of 1.5 ml were monitored for radioactivity. Most radioactivity was associated with the peak of lightest buoyant density (1.03 g/ml). After a 15 min labeling, radioactivity is associated with endosomes and plasma membrane fragments. Fractions around 1.03 g/ml density were pooled and used for acidification studies. The centrifugation profile was identical for controls and cells pretreated with DTNB or pCMBS.

Acridine Orange Assay for Endosome Acidification. The accumulation of acridine orange within acidic endosomes was measured as the difference between absorbance at 492 and 540 nm with a dual wavelength spectrophotometer. Endosomes (0.8 mg) were added to the acidification buffer in a water-jacketed cuvette and equilibrated at 37°C for 20 min prior to the addition of 1 mM ATP. Absorbance was measured over a period of 7 or 8 min, either immediately or 2 min following ATP addition. Endosomes were isolated from cells that had been exposed to 0.1 mM pCMBS.

Inhibition of DT by Addition of DTNB or pCMBS. Monolayers of CHO cells were exposed to concentrations of 0, 100, and 500 ng/ml DT for 2 h at 37°C in the presence or absence of the sulfhydryl inhibitors. After washing, they were reincubated in a medium containing radioactive

amino acids to measure the toxin's effect on protein biosynthesis. In the absence of inhibitors, 100 and 500 ng of DT caused a marked, dose-dependent decrease of amino acid incorporation (Table I). Addition of 1.0 mM DTNB almost totally prevented this inhibition (93.5 and 85.9%, respectively), and 0.1 mM pCMBS reduced the toxin's effect by 68 to 63%, respectively. At these concentrations, the two inhibitors had negligible effect on cell viability and, in the absence of toxin, decreased amino acid incorporation by 13.6 and 13.3%, an effect that was corrected for in Table I. In prior experiments testing the reductive cleavage of [^{125}I]tyn-SS-PDL, both DTNB and pCMBS had been used at concentrations of 0.1 mM. At that concentration, DTNB had no effect on the cytotoxicity of 500 ng/ml DT, and at 0.3 and 0.5 mM it inhibited the effect by only 1 and 4.5%, respectively.

TABLE 1

Effect of sulfhydryl blockers on cytotoxicity of DT and Ricin

Cytotoxicity was measured as decrease in ^{14}C-labeled amino acid incorporation into cellular proteins.

Toxin	Inhibitor	Relative incorporation	Reversal of toxicity
ng/ml	mM	% of control	%
DT			
0		100%	
100	0	42.8±2.1	
	1.0 DTNB	93.3±3.2	93.5
500	0	12.3±2.7	
	1.0 DTNB	87.6±7.9	85.9
100	0	32.7±8.8	
	0.1 pCMBS	78.4±3.2	67.9
500	0	23.5±3.3	
	0.1 pCMBS	71.3±2.0	62.5
Ricin			
0		100%	
100	0	59.4±10.3	
	1.0 DTNB	32.8±7.3°	--66.5°
500	0	16.8±1.5	
	1.0 DTNB	9.5±1.2°	-8.8°
100	0	42.5±4.4	
	0.1 pCMBS	27.0±8.8°	-2.0°
500	0	16.9±3.2	
	0.1 pCMBS	19.6±1.4	3.2

Since DTNB and pCMBS slightly inhibited amino acid incorporation in the absence of toxins the control

measurements (no toxin) were made in the presence of the respective inhibitors. The absolute incorporations were 7738 ± 885 cpm/mg of protein and 6037 ± 66 cpm/mg of protein for the DTNB and the pCMBS controls, respectively. Ricin, which like DT requires reductive cleavage of its interchain disulfide bond for activity was tested under the same conditions. Comparable dose-dependent inhibitions of amino acid incorporations were observed in the presence of ricin but were not prevented by DTNB and pCMBS. In fact, these reagents significantly enhanced the effect of the lower dose of ricin (Table 1).

In view of the prior finding that DTNB and pCMBS inhibited the reductive cleavage of a surface-bound disulfide conjugate (1), it is reasonable to assume that they similarly block the cleavage of a disulfide bond in membrane-bound DT. The loss of DT cytotoxicity reported in Table I could, however, also be ascribed to inhibitory effects of DTNB and pCMBS on DT activity prior to binding to DT receptors, on the internalization process itself, or on the process of endosome acidification. These three possibilities were ruled out as follows.

<u>The inhibition of Diphtheria Toxin (DT) is not due to an inhibitory effect on diphtheria toxin activity prior to binding to diphtheria toxin receptors</u>

DT (10µg/ml) was incubated in serum-free medium containing 1.0 mM DTNB for 2 h at 37°C, after which the mixture was diluted 100-fold with medium and tested as in Table I. The dilution brought DTNB to a concentration which, as mentioned above, is much to low to inhibit the cytotoxicity of 100 ng/ml DT. After this pretreatment, DT was fully cytotoxic, indicating that DTNB had not altered the ability of DT to bind to its receptor or to be internalized and activated. In similar experiments, pretreatment with pCMBS also failed to alter the

cytotoxicity of DT. (See Ryser et al., 1991). The further possibility that the inhibitors might alter the affinity of the receptor for DT could not be tested because the high level of nonspecific binding makes DT binding measurements difficult to interpret in CHO cells. The small size of the inhibitors, however, makes steric hindrance unlikely, and nothing in their structure suggests that they would mimic the B-chain binding site.

The inhibition of Diphtheria Toxin is not due to an inhibitory effect on the receptor-mediated endocytosis process itself.

The possibility that DTNB and pCMBS might inhibit receptor-mediated endocytosis was tested by measuring the cellular uptake of [^{35}S]Man-6-P enzymes) mediated by the Man-6-P receptor. At the concentration used in Table I, the two inhibitors failed to inhibit the endocytic uptake of this specific ligand (see Ryser et al., 1991), also indicating that they failed to inhibit its binding.

The inhibition of Diphtheria Toxin (DT) is not due to an inhibitory effect on the endosome acidification process.

The possibility that DTNB and pCMBS might protect cells from DT by interfering with endosome acidification was tested in two ways. In the first series of experiments, the pH of endosomes was increased by treating cells with NH_4Cl prior to measuring the uptake of [^{35}S]Man-6-P enzymes. Under physiologic conditions, the internalized enzyme-receptor complex dissociates at the acid pH of endosomes, allowing the Man-6-P receptor to recycle to the cell surface and the ligand to be carried to lysosomes. In NH_4Cl-treated cells, ligand and receptors fail to dissociate, leading to the recycling of an occupied receptor and to a decrease in enzyme uptake.

NH_4Cl pretreatment decreased the uptake of [^{35}S]Man-6-P enzymes, as expected, while DTNB and pCMBS did not (see Ryser et al., 1991). This rules out that the two inhibitors would markedly increase endosomal pH at concentrations that abolish DT cytotoxicity.

In the second set of experiments, the ability of endosomes to acidify their content by means of their ATP-dependent proton pump was tested directly by measuring the accumulation of acridine orange in the acidified vesicles. Cells were exposed at 1.0 mM DTNB or 0.1 mM pCMBS for 15 min at 37°C, together with [^{125}I]PLL used as a marker for endosomes. Isolated endosomes were incubated in the presence of ATP to measure the time-dependent change in acridine orange absorbance. The endosomes were fully capable of acidifying their content (see Ryser et al., 1991). It is reasonable to extrapolate that exposed intact cells remain capable of acidifying their endosomes.

EXAMPLE 3 Demonstration that HIV Infection of Cells is Inhibited by the Sulfhydryl Blocker (DTNB) in an Assay Measuring the Level of P24 in Human H9 Cells Exposed to HIV (HTLV 3B)

The H9 and C8166 cell lines as well as HIV/HTLV IIIB were obtained from Dr. Martin Hirsch, Massachusetts General Hospital, Boston, with permission of Dr. Robert Gallo, NIH.

Measurement of HIV infection

H9 cells grown in R20 medium were resuspended in serum free medium and exposed for 2 h at 37°C to approximately 1 x 10^7 $TCID_{50}$ units per 10^6 cells. Unbound virus was then removed following centrifugation and cells were plated in 24-well culture plates (8 x 10^5 cells/well) and incubated for 7 d at 37°C in R20 growth medium. Cell-

free culture supernatants containing 0.5% Triton X-100 were then assayed for their content in p24 viral protein using the HIV-1 p24 Core Profile ELISA (DuPont-NEN Research Products, Boston). The Kinetical program from Biotek Instrument, Inc., Winooski, VT was used for a kinetic as well as an end point analysis of p24. Viral titer was assayed as described by hartshorn, et al., Antimicrob. Agents Chemother., 31:168-172 (1987) and carried out in sixtuplets using C8166 cells as targets.

Results and Discussion

Human lymphoid H9 cells were exposed for 30 min at 37°C to 2.5 mM DTNB in serum-free medium to which HIV was added for 2 h at 37°C. After removal of both DTNB and unbound virus and incubation for 7 days in growth medium, the cells were harvested and their supernatant tested for its content of p24 viral protein. As shown in figure 5(B), no measurable level of p24 was detected in the supernatant of cells treated with 2.5 mM DTNB, in either of the five experiments performed. The supernatants of one experiment represented in Figure 5(B) were tested in a viral titer assay to compare the level of HIV p24 antigen and the titer of infective HIV released in the growth medium. The virus titer was determined by infecting C8166 cells with supernatants of known P24 contents. DTNB was present half an hour prior and during the 2-hour exposure to the virus. The p24 values and viral titers were as follows:

		HIV-Titer ($TCID_{50}$)		
Inhibitor	p24 (% of Control	Controls	Test	% of Control
DTNB (2.5mM)	<1	1.7×10^7	$<0.8 \times 10^2$	<0.001

A distinct dose-effect relationship was seen in all experiments testing more than one dose in the range of 0.25 and 2.5 mM DTNB as shown below:

DTNB (mM)	Inhibition (% of Control)
2.5 mM (n=4)	0.12 ± 0.11
1.0 mM (n=5)	14.8 ± 7.6
0.50 mM (n=2)	35.7
0.25 mM (n=2)	55.3

To test whether this inhibition might be due to an inactivation of HIV, a virus excess was exposed to 5.0 mM DTNB in serum-free medium for 2.5 h at 37°C and the virus-DTNB mixture was diluted to bring DTNB to a concentration known to have no effect on disulfide reduction. Virus treated in this way was only moderately less infective to H9 cells than untreated virus, as determined by the release of p24 antigen (Fig. 5(C)). Even though DTNB is membrane-impermeant, the possibility that it might indirectly inhibit virus growth in virus infected cells was tested by exposing H9 cells first to HIV virus for 2 h, then to 5 mM DTNB for 2.5 h before transfer to regular growth medium for 7 d. As shown by Fig. 5(D) this treatment did not suppress virus infection. These data indicate that, in order to be inhibitor, DTNB must be present at the time of initial virus-cell interaction. The results presented are consistent with the view that DTNB prevents cell surface sulfhydryls from interacting with disulfides in envelope proteins of attached virus and that disulfide cleavages that normally result from such interchanges are critical for HIV infection.

It has been generally thought that gp120 interaction with CD4 induces conformational changes that ultimately allow fusion of the virion envelope with the cell membrane and that the trigger of HIV fusion may be elicited by the

target cell. We propose that the triggering event is the reduction of disulfide bonds in gp120. Subtle conformational changes in the Sindbis envelope detected upon virus attachment were found to be similar to those caused by virus treatment with reducing agents. Thus, reduction of disulfide bonds in envelope proteins would appear to follow attachment of both HIV and Sindbis virus. Blocking this process with DTNB inhibited Sindbis infection and, as shown in Fig. 5(B), suppressed HIV infection.

These experiments lead to the following conclusions:

1. The membrane impermeant sulfhydryl inhibitor DTNB has a dramatic inhibitory effect on the HIV infection of H9 cells as measured either by the release of p24 or of infectious virus by infected cells.
2) DTNB, by blocking critical cell surface sulfhydryls, blocks the disulfide interchange between free sulfhydryls on the cell surface and structurally important disulfides present in the binding domain (and possibly the V3 loop of gp120) and thereby prevents the cleavage of critical disulfide bonds which must occur to initiate conformational changes leading to the cellular penetration of the cell-bound virus.
3) The cellular sulfhydryls engaged in the reductive mechanism of cell-bound viral proteins must be located in the immediate vicinity of the cellular receptor for HIV, known as the CD4 receptor.

Comparison of These Experimental Results with the DTNB Effect Observed in Non-Viral Systems in CHO Cells

The inhibition of viral P24 synthesis induced by 1.0 mM DTNB in the viral system is quantitatively very similar

to the inhibition of diphtheria toxin cytotoxicity by the same DTNB concentration in CHO cells (85 to 93%). The latter inhibition can be attributed to the blocking, by DTNB, of the cleavage of the disulfide bond that holds together the two chains of the surface-bound diphtheria toxin (DT). We described a membrane-associated mechanism in CHO cells capable of cleaving the disulfides of membrane-bound macromolecules (Feener, E.P., et al., JBC, 265:18780-18785 (1990)). By analogy we suggest that DTNB inhibits viral infection by blocking the same membrane-associated disulfide cleavage mechanism, hence that cleavage of critical disulfide bonds in the outer proteins of cell-bound HIV virus is required for viral infection.

Experiments Testing the Cytotoxicity of DTNB to H9 Cells

H9 cells were exposed to 3 and 10 mM DTNB for 2 hr at 37° in serum-free Eagle's medium, after which the DTNB-containing medium was washed off and replaced by RPMI growth medium. Cellular integrity was tested after 1 and 2 days of growth using the tetrazolium cytotoxicity assay (MTT assay) based on measuring the oxidative capacity of mitochondria as an expression of cellular integrity. (Mosmann, T. J., Immunol. Meth. 22:1759-1770 (1983). Results are shown below:

	Cellular Integrity After	
	1 Day	2 Day
Controls	100%	100%
DTNB 3.0 mM	103.9%	81.6%
DTNB 10 mM	88.2%	92.1%
pCMBS 0.1 mM	24.8%	31.5%
pCMBs 0.3 mM	0%	1.1%

pCMBS which, like DTNB, is a membrane impermeant disulfide blocker, was used as a positive control for cytotoxicity, since we found it to be cytotoxic to human cells (HeLa

cells in culture). It is worth noting that in this control experiment, the DTNB concentration was four times the concentration used in the viral experiment, and present over a longer period of time (120 min vs. 90 min). The lack of visible and measurable cytotoxicity of DTNB support the data of Fig 5D in excluding the possibility that the marked inhibition of P24 synthesis seen in the HIV work would be due to DTNB cytotoxicity to H9 cells.

A further control experiment was the demonstration that the DTNB concentrations used did not inactivate the virus in vitro and was carried out in the following manner. HIV at 100-fold the multiplicity used for infecting H9 cells was exposed to 2.5 mM DTNB for 90 min at 37° C. and was then diluted 100-fold to bring the concentration of DTNB below infection-inhibiting concentrations. After that dilution, DTNB-treated HIV remained fully infective. Inactivation of the virus by DTNB would inhibit infectivity and might indeed cause an inhibition of P24 synthesis.

EXAMPLE 4 Inhibition of Reductive Cleavage of the Model Compound ^{125}I-tyn-SS-PDL by Bacitracin

Prior to testing the effect of Bacitracin (purchased from Sigma), an inhibitor of PDI, on the cleavage of surface-bound ^{125}I-tyn-SS-PDL, it was important to ascertain in a cell free system that ^{125}I-tyn-SS-PDL was susceptible to cleavage by PDI. Therefore, in a preliminary experiment, ^{125}I-tyn-SS-PDL was used as a substrate in the glutathione: insulin transhydrogenase assay described previously in this example. The following table demonstrates that such is indeed the case.

Substrate	PDI	Cleavage %	PDI-induced Cleavage (Increase %
^{125}I-tyn-SS-PDL	-	24.8	-
	+	49.7	25%
^{125}I-insulin	-	18.6	-
	+	48.8	29.1

Cleavage was measured during a 30 minute exposure at 37°C and is expressed as percentage of the added radioactivity. Compared to the conventional assay for PDI activity, the GSH concentration was reduced from 250 μM to 100 μM.

For comparison, the table gives values of insulin reduction, measured in the conventional assay for PDI activity, using insulin as substrate. The PDI induced cleavage was comparable using either substrate.

PDI cleaved 25% of the conjugate, a value that compares favorably with the cleavage of ^{125}I-insulin (29%) by the same amount of PDI. On the basis of these data, it can be expected that surface-bound ^{125}I-tyn-SS-PDL will also be susceptible to cleavage by membrane-associated PDI. This is indeed demonstrated by Figure 4.

The experiments used to determine whether bacitracin inhibits the reductive cleavage of the model compound were essentially the procedures described by Feener et al., J. Biol. Chem., 265: 18780-18785, 1990, except bacitracin was used instead of DTNB or pCMBS. Cells were labelled with ^{125}I-tyn-SS-PDL at 0°C for 60 min, washed several times with cold medium and reincubated for 30 min at 37°C in serum free medium containing various concentrations of bacitracin. At that time cells were solubilized and the acid soluble radioactivity of cells and medium was measured and expressed as percent of the initial

radioactivity (chase time = 0). Initial O-time values were measured separately for each bacitracin concentration.

This figure shows that bacitracin inhibits the membrane-associated disulfide cleavage of the surface-bound conjugate in a dose-dependent manner. Between 0.3 and 3.0 mM, the inhibition increased from 40 to 76%, an increment comparable to the one seen in the inhibition of PDI activity in vitro (60 to 95%, Fig. 2). Surprisingly, we found that acid-soluble cell-bound radioactivity was generated during the 60 min labeling at 0°C and that this cleavage was also decreased in dose-dependent fashion by bacitracin present during labeling. This bacitracin-induced inhibition of cleavage is represented in the upper curve of Fig. 4. It indicates that some surface-associated cleavage occurs at a temperature known to arrest endocytosis, and thus that bacitracin can exert an inhibitory effect at the cell surface. It is difficult to conceive how these bacitracin effects could be non-specific artifacts. Exposure of Chinese hamster ovary cells to 3.0 mM bacitracin for 2 h at 37°C did not cause cell damage in the MTT cytotoxicity assay (Mosmann, T.J., Immun. Met., 22:1759-1770 (1983)). Moreover, a bacitracin leakage of intracellular PDI into the medium would, if anything, increase the reductive cleavage of membrane-bound ^{125}I-tyn-SS-PDL. The bacitracin-induced inhibitions seen in Fig. 4 must therefore be due to a specific inhibition of surface-associated PDI. That the inhibition of reduction occurs at the cell surface is further demonstrated by the fact that the zero-values measured separately for each bacitracin concentration also showed a dose-dependent decrease in cleavage under conditions that abolish endocytosis (See Fig. 4, upper curve).

EXAMPLE 5 Inhibition of the diphtheria toxin by addition of bacitracin, a known inhibitor of PDI

Bacitracin, purchased from Sigma, was used instead of DTNB or pCMBS in experiments identical to those described in Example 2. Bacitracin caused a dose-dependent inhibition of DT cytotoxicity in the range of 0.3 to 3.0 mM, concentrations that were non-toxic to CHO cells. Fig. 1 expressed the data as percent protection from DT cytotoxicity. At 3.0 mM, bacitracin afforded 80% protection from DT cytotoxicity. As shown in Example 6 (Fig. 2) 3.0 mM bacitracin caused a 95% inhibition of PDI in the in vitro insulin reduction assay. Bacitracin alone (3.0 mM) caused a slight inhibition of protein biosynthesis (91 ± 5.9% of controls, n=21), which was taken into account in the calculations. The magnitude of protection obtained with a given dose of bacitracin was influenced by the initial level of diphtheria toxin cytotoxicity, which in the three experiments of Fig. 1 averaged 36% of controls. In a larger series of experiments, protection was found to be greatest when DT alone reduced protein biosynthesis between 25 and 50% of control values. A similar correlation was noted in the protective effect of DTNB and pCMBS. The postulate that this inhibition of DT cytotoxicity is due to an inhibition of PDI is supported by the dose-effect relationships of bacitracin in all three systems, (see Fig. 2, Fig. 4, lower curve, and Fig. 1), in which the ID_{50} obtained from the dose-effect curves were as follows:

Test System	ID_{50} (mM)
PDI activity *in vitro*	0.25
Cleavage of the Disulfide Conjugate	0.62
Activation of Diphtheria Toxin	0.90

This concordance is quite remarkable considering that PDI acts in these cases on three different substrates and in systems with different end points. These data strongly suggest that the sulfhydryls critically involved in the reductive activation of DT are cysteine residues of PDI and that the reductive function of the plasma membrane we described is catalyzed by PDI present at the cell surface.

As had been found with the non-permeant sulfhydryl blockers, bacitracin did not reduce the cytotoxicity of ricin, and in fact, slightly increased it. As discussed elsewhere, this observation is consistent with the currently accepted view that reductive cleavage of ricin's interchain disulfide occurs in the trans-Golgi network or the Golgi apparatus, i.e., at a site not accessible to membrane impermeant PDI inhibitors. HeLa cells, the only cell type other than CHO tested in this fashion, were also consistently protected by bacitracin against DT cytotoxicity.

The marked differences in structural and biochemical properties of DTNB and bacitracin suggest that they may inhibit PDI by different mechanisms. It was of interest, therefore, to test whether the two categories of inhibitors would have additive effects. In two experiments carried out at different levels of DT cytotoxicity, the average protection afforded by 3.0 mM bacitracin and 1.0 mM DTNB given alone or in combination, are shown below:

Inhibitors	% Inhibition of DT Cytotoxicity
Bacitracin alone (3 mM)	38.8 ± 16.8
DTNB alone (1.0 mM)	23.8 ± 17.0
Both Inhibitors Together	64.4 ± 14.2

Similar results were obtained when pCMBS was used instead of DTNB. Bacitracin alone, pCMBS alone (0.1mM) and both inhibitors given together caused 41 ± 3.2, 29.5 ± 17 and 75.6 ± 32% inhibition of DT cytotoxicity, respectively.

EXAMPLE 6 Inhibition of PDI Activity by Bacitracin, Three Membrane Impermeant Blockers of Sulfhydryls and by Anti-PDI Antibodies

Additional materials used were: PDI isolated from calf liver as described by Hilson, D.A., et al., Meth. in Enzymology, 107: 281-294 (and was 90 to 95% pure by electrophoresis) and monobromotrimethylammoniobimane (thiolyte MQ) from Calbiochem. Lyophilized ascites fluid containing anti-PDI monoclonal antibodies RL77 and HP13 and the parent hybridoma cells were a gift from Dr. Charlotte S. Kaetzel, Case Western Reserve University School of Medicine (Kaetzel, S. et al., Biochem. J., 241:39-47 (1987). For some experiments the antibody containing ascites fluid or conditioned medium were purified by protein G affinity chromatography (Mab Trap G Kit from Pharmocia-LKB, Piscataway, NJ).

PDI activity was measured by the glutathione: insulin transhydrogenase assay described by Carmichael, D.F., et al., J. Biol. Chem., 252: 7163-7167, (1977), as modified by Kaetzel C. S., et al., Biochem. J., 241: 39-47 (1987). The assay was further modified by reducing the total volume to 0.5 ml, extending the reaction time to 30 min, and stopping the reaction with 10 mM NEM, prior to TCA-precipitation. The amount and radioactivity of ^{125}I-insulin were 50 μg and 10^5 CPM/0.5 ml. Under these conditions, PDI had a specific activity of 5,200 U/mg in the standard 5 min assay. PDI activity was calculated as the difference between acid-soluble radioactivity generated in a 30 min incubation at 37°C in presence or

absence of PDI. Controls were run to determine acid soluble radioactive contamination of the ^{125}I-insulin preparations (usually 2 to 6%). Assays were carried out in the presence and absence of aprotinin to monitor possible proteolytic cleavage of ^{125}I-insulin. They showed that insulin proteolysis was not a factor in our assay condition. To test the effect of Bacitracin, DTNB and pCMBS, these inhibitors were added to the reaction mixture a few minutes prior to PDI addition. Fig. 2 shows that the three sulfhydryl blockers (DTNB, pCMBS and thiolyte MQ) inhibit PDI activity to a comparable extent and have dose-inhibition curves of comparable slopes. Bacitracin at 3.0 mM inhibits PDI activity by 95%. Its dose-inhibition curve, however, has a different slope consistent with the fact that it inhibits PDI by a mechanism different from the blocking of PDI's sulfhydryl group. As a control, the activity of the enzyme thiorexdoxin, which like PDI catalyzes disulfide reactions and occurs in plasma membrane fractions of mammalian cells, was measured in the presence of bacitracin. The activity of thiorexdoxin was determined in the turbidimetric assay described by Holmgren, A., J. Biol. Chem., 254: 9627-9632. The activity of thiorexdoxin was not inhibited by bacitracin.

Effect on Anti-PDI Antibodies on the Activity of PDI in the Insulin Reduction Assay

Different concentrations of the HP13 and RL77 antibodies directed against human and rat PDI, respectively were added to the reaction mixture at the same time as PDI. As shown in Fig. 3 and Table 2, both antibodies decrease PDI activity in a dose-dependent manner. The upper curve shows results from three experiments using an antibody (HP13) directed against human PDI. The lower

curve shows results from 7 experiments, using an antibody (RL77) directed against rat PDI. The data show that both antibodies are inhibiting to calf liver PDI. The observation that RL77 could cause only 49% inhibition of PDI activity is consistent with the finding of Kaetzel, C.S., et al., Biochem. J., 241: 39-47, and with their suggestion that these antibodies bind at or near only one of the two active sites of PDI.

EXAMPLE 7 Effect of Anti-PDI Antibodies on Cleavage of Surface-Bound ^{125}I-tyn-SS-PDL

The same antibodies used in Example 6, as well as a third antibody (RL90), were used to test their ability to inhibit cleavage of ^{125}I-tyn-SS-PDL.

Three experiments to test the effect of anti-PDI antibodies were carried out using a procedure similar to that used to obtain the data of Figure 4. The cells were grown in 24-well plates rinsed with ice-cold medium and preincubated for 30 min at 0°C, in serum-free Eagle's medium containing anti-PDI antibodies or irrelevant IgG and 25mM Hepes buffer at pH 7.4, in a total volume of 0.3ml prior to labelling with ^{125}I-tyn-SS-PDL. The antibodies were those used in the PDI-inhibition assay (Figure 3). ^{125}I-tyn-SS-TDL (lug/ml, 1.8×10^5 cpm/well) was then added to each well to label the surface of the cells for 30 min at 0°C. The cells were washed twice and reincubated in prewarmed medium (37°C) containing antibodies or irrelevant IgG for a 30 min incubation. A further modification was the omission of bovine serum albumin from the incubation medium. When matched against the effect of comparable amounts of irrelevant IgG, all three antibodies had marked inhibitory effects. The affinity purified antibodies RL77 and HP-13 at concentrations of 25 ug/well caused inhibitions of 43% and

51%, respectively. RL90 tested in reconstituted ascites fluid (0.6 mg proteins/well) caused a 62% inhibition. These data show that the surface-associated reductive process we described can be inhibited by anti-PDI antibodies, providing further evidence that the process is catalyzed by the enzyme PDI. See Table 2.

TABLE 2

Inhibition of PDI activity by monoclonal anti-PDI antibodies in the insulin reduction assay

Concentrations (ug protein/ml)	RL77 (% decrease)	HP13 (% decrease)
10	9.7 ± 6.8 (5)*	14 ± 6.5 (3)
20	10 ± 13 (4)	23 ± 9.7 (2)
50	32 ± 11 (10)	30 ± 11 (3)
100	41 ± 11 (2)	---
200	49 (1)	---

*Numbers of experiments are given in parenthesis.

EXAMPLE 8 Inhibition of the Diphtheria Toxin by Anti-PDI Antibodies

The same antibodies used in Example 7 were tested for an inhibitory effect on DT toxicity in 4 experiments, similar to those carried out in Example 2, in which DT alone depressed amino acid incorporation into proteins to 47% control values. Cells were preexposed to medium containing antibodies as described in Example 7, except that the antibodies were given as aliquots of reconstituted solutions of lyophilized ascites fluid containing 10 mg protein/ml (0.84 mg/well). Controls contained the same protein amount as a mixture of irrelevant IgG and bovine serum albumin at the ratio found

in ascites fluid (1:9). DT (50 ul) was added in each well to the antibody-containing medium, the culture plates were incubated for 2 hours at 37°C and processed as described in Ryser, J.J.-P., et al., Biol. Chem., 266: 18439-18442 (1991). It was found in previous experiments that simple pretreatment of cells with DTNB for 1 hour at 0°C did not inhibit the cytotoxicity caused by a subsequent 2 hour exposure to DT at 37°C, and that pretreatment with pCMBS led only to partial inhibition. Anti-PDI antibodies were therefore present both during preincubation at 0°C and incubation at 37°C when measuring DT cytotoxicity.

Compared to the effect of identical concentrations of irrelevant IgG, all three antibodies caused significant inhibition of DT cytotoxicity (Table 3). Compared to the IgG control (7.1 inhibition), the levels of significance for the inhibitions were $p<0.05$ for RL77, $p=0.001$ for RL90 and $p<0.05$ for HR13). See Table 3. The inhibition of amino acid incorporation by DT varied from 41% to 53% with a mean of 47% ± 4.9% (n=4). As noted with bacitracin, the magnitude of inhibition of DT-cytotoxicity was influenced by the level of initial DT-cytotoxicity. In 3 experiments in which RL77 was tested at 2 or more concentrations, its effect was clearly dose-related. See Figure 6.

TABLE 3

Effect of anti-PDI antibodies on DT cytotoxicity

DT	IgG or antibodies	Relative incorporation	Inhibition of toxicity
-	-	% of control	%
+	-	100	
+	-	47 ± 4.9	0
+	IgG	51 ± 6.1	7.1 ± 3.7
+	RL77	75 ± 15	54 ± 24
+	RL90	94 ± 10	85 ± 12
+	HR13	80 ± 20	64 ± 33

EXAMPLE 9 Inhibition of HIV by Anti-PDI antibodies and Bacitracin

The fact mentioned earlier that inhibitory effects on surface-associated disulfide reduction caused by DTNB could be reproduced with two inhibitors of PDI suggested that anti-PDI antibodies and bacitracin might, likewise, interfere with HIV infection. Monoclonal antibody HP13 directed against human PDI was added for 30 min at 37°C to H9 cells suspended in serum-free medium, to which HIV was then added for a 2 h infection period at 37°C. Control cells were exposed to the same concentration of an irrelevant IgG. As shown in Fig. 7(B) exposure to anti-PDI antibodies reduced HIV infection to 17 ± 3.4% of controls. This inhibition (83 ± 3.4%) is greater than had been expected, since the inhibitory effect of the same antibody on PDI activity *in vitro* was never found to exceed 50%. (Kaetzel et al., Biochem. J. 241:39-47 (1987). This suggests that the antibody might bind to

only one of the two active sites of soluble PDI, and that only that one site is exposed in membrane-associated PDI. Supernatants of cells that had been exposed to anti-PDI antibody and in which p24 levels were decreased to 20.5% of controls (average of quadruplicates) were tested for their titer of infectious virus as described in Example 3. The titer was decreased to less than 10% of controls as shown below:

HIV-Titer ($TCID_{50}$)

Inhibitor	p24 (% of Control)	Controls	Test	% of Control
HP-13 Anti-PDI Antibody	20.5	6.0×10^2	5.0×10^1	8.3

Similar inhibitions were obtained with the monoclonal anti-PDI antibody RL90 directed against rat PDI. At a concentration of 100 µg/ml, RL90 caused a 17% inhibition of PDI activity *in vitro*. At 290 µg/ml it inhibited HIV infection by 90 ± 4.4% (p24 release). The antibody against rat PDI, thus, crossreacts with human PDI.

Bacitracin was tested at a concentration (3.0 mM) that had been found to inhibit the cytotoxicity of DT in CHO cells. When H9 cells were exposed to bacitracin for 30 min prior to and during the 2 h infection period, the subsequent release of p24 proteins into their culture medium was significantly decreased (Fig. 7C). In an experiment in which the p24 level was decreased to 1.5% of controls, a corresponding viral titer assay was performed as in Example 3 and the viral titer was reduced to 0.13% of quadruplicate controls as shown below:

HIV-Titer ($TCID_{50}$)

Inhibitor	p24 (% of Control	Controls	Test	% of Control
Bacitracin (3.0mM)	1.5	$>1.0x10^7$	$1.4x10^4$	<0.1

Bacitracin is transported poorly into cells and evidence was provided that its effect on the cleavage of membrane-bound [^{125}I]-tyramine-SS-poly-D(lysine) does not require cellular uptake. It can be assumed therefore that, like DTNB and anti-PDI antibodies, bacitracin exerts its effect on the plasma membrane of H9 target cells. The possibility that DTNB and bacitracin would interfere with the attachment of HIV to its cellular receptor has not been experimentally excluded, but appears unlikely, since it would require that two molecules as similar as DTNB and bacitracin would have similar effects on virus binding, and would decrease in similar fashion the attachment of HIV and of DT to their respective cell surface receptors.

It is evident from comparing Figs. 5 and 7 that DTNB is a more powerful inhibitor than either bacitracin or anti-PDI antibodies. A similar trend can be seen in the relative effect of these inhibitors on ^{125}I-tyn-Ss-PDL cleavage and DT cytotoxicity. Of the three classes of inhibitors, however, the monoclonal antibodies are the most specific. The fact that DTNB is more powerful may be due to its ability to block membrane thiols required to regenerate the oxidized PDI that results from the PDI-catalyzed thiol:disulfide interchange.

Altogether our data suggest that HIV infection can be inhibited by preventing a sequence of events initiated by the cleavage of viral disulfide bonds at the cell surface. The data and considerations that support this view can be summarized as follows: 1) Our results indicate that HIV infection is inhibited by three different kinds of

reagents that, at comparable doses, have each been shown to inhibit disulfide cleavage in other membrane-bound macromolecules. Their effectiveness ranks similarly in HIV infection and in the other two systems. 2) Similar inhibitions are observed when thiol:disulfide interchange is prevented by blocking membrane thiols belonging to PDI (Fig 5 and 7) or by disrupting viral disulfides prior to HIV-cell interaction (Fig 9 and Example 11). 3) gp120, the viral protein involved in the binding of HIV to its cell receptor, is known to contain 18 conserved cysteine residues forming 9 disulfide bonds. Three of them are situated in, or close to, the domain that binds to CD4 and are plausible substrates for PDI-catalyzed interchanges following HIV-CD4 interaction. 4) Cleavage of protein disulfides is capable of initiating conformational changes comparable in importance to the pH-induced conformational changes associated with the penetration of other viruses. 5) The three classes of agents which in our experiments inhibit HIV infection, also inhibit the activity of PDI in vitro at comparable concentrations. 6) The multi-functional enzyme PDI, although predominantly located in the endoplasmic reticulum, is also exposed at the surface of mammalian cells where it is capable of initiating thiol-disulfide interchanges.

While not excluding participants other than PDI in this surface-associated reductive process, the concurrence of the above findings strongly suggests that PDI plays a key role in the process of HIV infection, and implies that PDI is situated in close proximity to the CD4 receptor and is capable of reaching critical disulfides located within or close to the receptor-binding domain of gp120. Both the membrane-associated PDI and the critical envelope disulfides it cleaves offer novel targets for anti-HIV therapy. Neutralizing antibodies might be developed that are directed against epitopes containing these essential

disulfide bonds. More generally, these findings assign new importance to disulfide bonds in the function of viral envelope proteins and broaden the possibility that disulfide reduction might trigger the cellular penetration of other viruses.

EXAMPLE 10. Kinetics of ^{125}I-tyn-SS-PDL reduction by CHO cells following 16 hrs pretreatment with BFA (solid line) and untreated controls (dotted line).

CHO cells were preincubated for 16 hours in growth medium containing 1.0 μg/ml Brefeldin A (BFA). They were cooled to 0°C and pulse-labeled for 30 minutes at 0°C with ^{125}I-tyn-SS-PDL in presence of BFA, washed to remove unbound label, conjugate, and reincubated for 2 hours at 37°C in absence of labeled conjugate and in presence of BFA, at 37°C. The generation of ^{125}I-tyn-SH was measured after 30, 60 and 120 min of chase, and expressed as reduction of cell-bound ^{125}I-tyn-SS-PDL in percent of the cell-bound radioactivity measured at time 0. Figure 8 compares cells treated with BFA (upper curve) with untreated cells (lower curve). The largest difference in reductive cleavage is seen after 30 min, indicating that BFA influences the first phase of reduction occurring at the cell surface. Figure 8 represents an average of 3 experiments. When expressed in percent of the initial cell-bound radioactivity, the reductive cleavage measured between 0 and 30 min was 2.29 ±0.14 and 4.8 ±1.7 for controls and BFA treated cells, respectively. These results indicate that BFA pretreatment increases the reductive function of the plasma membrane.

EXAMPLE 11. Inactivation of HIV by DTT.

HIV/HTLV III B of very high titer (5 X 10^{12} TCID units/ml) was exposed for 2.5 hours at 37°C to increasing concentrations of the reducing agent dithiotreitol (DTT) in serum free minimal essential medium (MEM). The virus /DTT solution was serially diluted with MEM and virus aliquots were added to wells (sixtuplets) containing C8166 cells (See Example 3) in R20 growth medium, inoculating six wells for each concentration of DTT pretreatment. The multiwell plates were incubated for 7 days at 37°C to allow for virus expression and formation of syncytia. HIV infection was then scored by determining the virus titer at which heterokaryons were detected in 3 out of 6 wells as described by Hartshorn K.L. et al., Antimicrob. Agents Chemother., 31: 168-172 (1987). Serial dilution of the virus had brought the residual concentration of DTT to a level known to be non toxic to C8166 cells in all groups of 6 wells of determining virus titer. The points of Figure 9 give the percent of DTT-induced inhibition of HIV infectivity, based on the averages of 2-6 experiments. The results demonstrate that DTT inactivated HIV in a dose-dependent fashion up to a 100% inhibition of infectivity.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Trustees of Boston University
(B) STREET: 80 East Concord Street
(C) CITY: Boston
(D) STATE: MA
(E) COUNTRY: USA
(F) POSTAL CODE (ZIP): 02218

(ii) TITLE OF INVENTION: Method of Inhibiting Reduction of Disulfide Bonds

(iii) NUMBER OF SEQUENCES: 1

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 181 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Asn Ala Lys Thr Ile Ile Val Gln Leu Asn Gln Ser Val Glu Ile Asn
1 5 10 15

```
Cys Thr Arg Pro Asn Asn Asn Thr Arg Lys Ser Ile Arg Ile Gln Arg
            20                  25                  30

Gly Pro Gly Arg Ala Phe Val Thr Ile Gly Lys Ile Gly Asn Met Arg
        35                  40                  45

Gln Ala His Cys Asn Ile Ser Arg Ala Lys Trp Asn Asn Thr Leu Lys
    50                  55                  60

Gln Ile Asp Ser Lys Leu Arg Glu Gln Phe Gly Asn Asn Lys Thr Ile
65                  70                  75                  80

Ile Phe Lys Gln Ser Ser Gly Gly Asp Pro Glu Ile Val Thr His Ser
                85                  90                  95

Phe Asn Cys Gly Gly Glu Phe Phe Tyr Cys Asn Ser Thr Gln Leu Phe
            100                 105                 110

Asn Ser Thr Trp Glu Asn Ser Thr Trp Ser Thr Lys Gly Ser Asn Asn
        115                 120                 125

Thr Glu Gly Ser Asp Thr Ile Thr Leu Pro Cys Arg Ile Lys Gln Ile
    130                 135                 140

Ile Asn Met Trp Gln Glu Val Gly Lys Ala Met Tyr Ala Pro Pro Ile
145                 150                 155                 160

Ser Gly Gln Ile Arg Cys Ser Ser Asn Ile Thr Gly Leu Leu Leu Thr
                165                 170                 175

Arg Asp Gly Gly Asn
            180
```

CLAIMS

The invention claimed is:

1. Use of a) a sulfhydryl blocker; b) a protein disulfide isomerase-specific inhibitor; or c) a combination of (a) and (b), for the manufacture of a medicament to effect inhibition of the reduction function of a eukaryotic cell membrane.

2. Use of a) a sulfhydryl blocker; b) a protein disulfide isomerase-specific inhibitor; or c) a combination of (a) and (b) for the manufacture of a medicament to inhibit protein disulfide isomerase for inhibiting protein disulfide isomerase-catalyzed reductive cleavage of disulfide bonds in the binding area of specific macromolecular ligands at the cell surface membrane of a eukaryotic cell.

3. The use of Claim 1 or Claim 2, wherein the sulfhydryl blocker is 5,5′-dithiobis-2-nitrobenzoic acid, p-chloromer-curiphenylsulfonic acid or thiolyte MQ and the protein disulfide isomerase-specific inhibitor is an anti-protein disulfide isomerase antibody or bacitracin.

4. Use of a) a sulfhydryl blocker; b) a protein disulfide isomerase-specific inhibitor or c) a combination of (a) and (b) for the manufacture of a medicament for reducing the cytotoxicity to a eukaryotic cell of a cell surface membrane-bound toxin containing disulfide bonds, e.g. by inhibiting protein disulfide isomerase-catalyzed reductive cleavage of disulfide bonds in the cell surface membrane-bound toxin, so as to reduce

entry of the cell surface membrane-bound toxin into the cell and the cytotoxicity to the cell of the toxin.

5. The use of Claim 4 wherein the cell surface membrane-bound toxin is diphtheria toxin and the eukaryotic cell is a mammalian cell.

6. Use of a sulfhydryl blocker or a protein disulfide isomerase-specific inhibitor for the manufacture of a medicament for inhibiting infection of a human lymphoid cell by HIV, e.g. by inhibiting the reductive function of the human cell surface membrane which is required for the infective interaction of HIV.

7. The use of Claim 5 or Claim 6, wherein the sulfhydryl blocker is 5,5'-dithiobis-2-nitrobenzoic acid, p-chloromer-curiphenylsulfonic acid or thiolyte MQ and the protein disulfide isomerase-specific inhibitor is an anti-protein disulfide isomerase antibody or bacitracin.

8. Use of a sulfhydryl blocker, a protein disulfide isomerase-specific inhibitor or a combination thereof for the manufacture of a medicament for inhibiting infection of a human cell by HIV, e.g. by inhibiting protein disulfide isomerase-catalyzed reductive cleavage of disulfide bonds at the cell surface membrane of the human cell.

9. The use of Claim 8 wherein the sulfhydry blocker is DTNB or pCMBS and the protein disulfide isomerase-specific inhibitor is an anti-protein disulfide isomerase antibody or bacitracin.

10. Use, for the manufacture of a medicament for inhibiting passage across a eukaryotic cell membrane of all or a part of a cell membrane-bound macromolecule which includes disulfide bonds, cleavage of which is necessary for passage of the macromolecule across the cell membrane, of a sulfhydryl blocker, a protein disulfide isomerase-specific inhibitor or both, in an amount to inhibit protein disulfide isomerase-catalyzed reductive cleavage of disulfide bonds in cell membrane-bound macromolecules.

11. The use of Claim 10 wherein the macromolecule is selected from the group consisting of:
a) viruses, particularly retroviruses which contain a disulfide bond in their outer shell, e.g. HIV, and
b) toxins which contain a disulfide bond, cleavage of which is required for passage across a cell membrane, said toxins e.g. being selected from the group consisting of: diphtheria toxin, modeccin, pseudomonas toxin E, gelonin, toxic ribonucleases.

12. Use, for the manufature of a medicament for enhancing passage across a eukaryotic cell membrane of all or a part of a cell membrane-bound macromolecule which includes disulfide bonds, cleavage of which is necessary for passage of the macromolecule across the cell membrane, of a substance which enhances protein disulfide isomerase-catalyzed reductive cleavage of disulfide bonds in the macromolecule, in particular in a cell membrane-bound macromolecule, e.g. a hormone, selected from the group consisting of: growth hormone, interleukins, lymphokines, hematopoietic stimulating factors, specific epithelial, endothelial and fibroblast stimulating factors, transforming growth factors.

13. A sulfhydryl blocker or an inhibitor of protein disulfide isomerase, for use to reduce the ability of protein disulfide isomerase to cleave disulfide bonds at a eukaryotic cell surface membrane.

14. The use of Claim 13 wherein the sulfhydryl blocker is 5.5′-dithiobis-2-nitrobenzoic acid, p-chloromercuriphenylsulfonic acid or thiolyte MQ and the inhibitor of protein disulfide isomerase is an anti-protein disulfide isomerase antibody or bacitracin.

15. A method of determining whether an agent is an inhibitor of protein disulfide isomerase-catalyzed reductive cleavage of disulfide bonds in eukaryotic cell surface membrane-bound macromolecules, e.g. toxins or viruses, comprising the steps of:

a) combining a eukaryotic cell having bound to the cell surface membrane thereof a macromoloecule, referred to as a cell surface membrane-bound macromolecule and an agent to be assessed;

b) maintaining the product of (a) under conditions sufficient for protein disulfide isomerase-catalyzed reductive cleavage of disulfide bonds in cell surface membrane-bound macromolecules;

c) determining the extent to which the cell surfacemembrane-bound macromolecule crosses the cell surfacemembrane; and

d) comparing the determination made in (c) with the extent to which the cell surface membrane-bound macromolecule crosses the cell membrane in the absence of the agent to be assessed, wherein if the cell surface membrane-bound macromolecule crosses the cell surface membrane to a lesser extent in the presence of the agent to be assessed than in the absence of the agent to be assessed,

the agent to be assessed is an inhibitor of protein disulfide isomerase-catalyzed reductive cleavage of disulfide bonds in cell surface membrane-bound macromolecules.

16. An inhibitor of protein disulfide isomerase-catalyzed reductive cleavage of disulfide bonds in cell surface membrane-bound macromolecules identified by the method of Claim 22.

17. The use of Claim 6 wherein reductive cleavage of disulfide bonds is reductive cleavage of disulfide bonds in gp120, e.g. occurring in the V3 loop.

18. Use, for the manufacture of a therapeutic agent against protein disulfide isomerase-catalyzed reductive cleavage of disulfide bonds by contacting the human immunodeficiency virus with said agent, of a reducing agent, a specific inhibitor of the disulfide bond containing domains of human immunodeficiency virus or a combination thereof.

19. The use of Claim 18 wherein the reducing agent is dithiothreitol, β-mercaptoethanol, GSH, and/or the specific inhibitor is a monoclonal antibody directed against a critical disulfide bond-containing domain of human immunodeficiency virus.

20. Use of a reducing agent, a specific inhibitor of a disulfide bond-containing domain, or both for the manufacture of an inhibiting agent against cleavage of the disulfide bonds by the reductive function of the cell membranes for inhibiting infection of a eukaryotic cell by a virus by contacting the disulfide bonds of the virus which require cleavage for passage of the

virus across the cell membrane, prior to a virus-cell interaction.

21. The use of Claim 20 wherein the reducing agent is dithiothreitol, β-mercaptoethanol or GSH.

22. The use of Claim 20 wherein the specific inhibitor of the disulfide bond-containing domain comprises an antibody directed against disulfide bond-containing domains of the virus.

23. The use of Claim 20 wherein the virus is human immunodeficiency virus, hepatitis virus, herpes virus, papilloma virus, enterovirus or paramyxovirus.

24. A substance which inhibits cleavage of virus disulfide bonds by protein disulfide isomerase, for inhibiting infection of a eukaryotic cell by the virus, said virus, for example, being human immunodeficiency virus, hepatitis virus, herpes virus, papilloma virus, enterovirus or paramyxovirus.

25. The substance of Claim 24 which is a reducing agent, e.g. dithiothreitol, β-mercaptoethanol or GSH.

26. The substance of Claim 24 which is a specific inhibitor of the viral disulfide bonds, e.g. antibodies directed against disulfide bond-containing domains of the virus.

27. A method of inhibiting infection of a eukaryotic cell by a virus comprising contacting the disulfide bonds of the virus which require cleavage for passage of the virus across the cell membrane, prior to a virus-cell interaction, with:

a) a reducing agent in an amount sufficient to cleave the disulfide bonds of the virus;

b) a viral disulfide specific inhibitor in an amount sufficient to inhibit cleavage of the disulfide bonds by the reductive function of the cell membrane; or

c) a combination of (a) and (b) in an amount sufficient to cleave the disulfide bonds of the virus and inhibit cleavage of the disulfide bonds by the reductive function of the cell membrane

thereby inhibiting infectin of a eukaryotic cell by a virus.

28. The use of Claim 19 wherein the disulfide bond containing domains of human immunodeficiency virus is in gp120 of human immunodeficiency virus.

29. The use of Claim 28 wherein the critical disulfide bond containing domain of the gp120 of the human immunodeficiency virus is in the C3 region, the C4 region or the V3 loop.

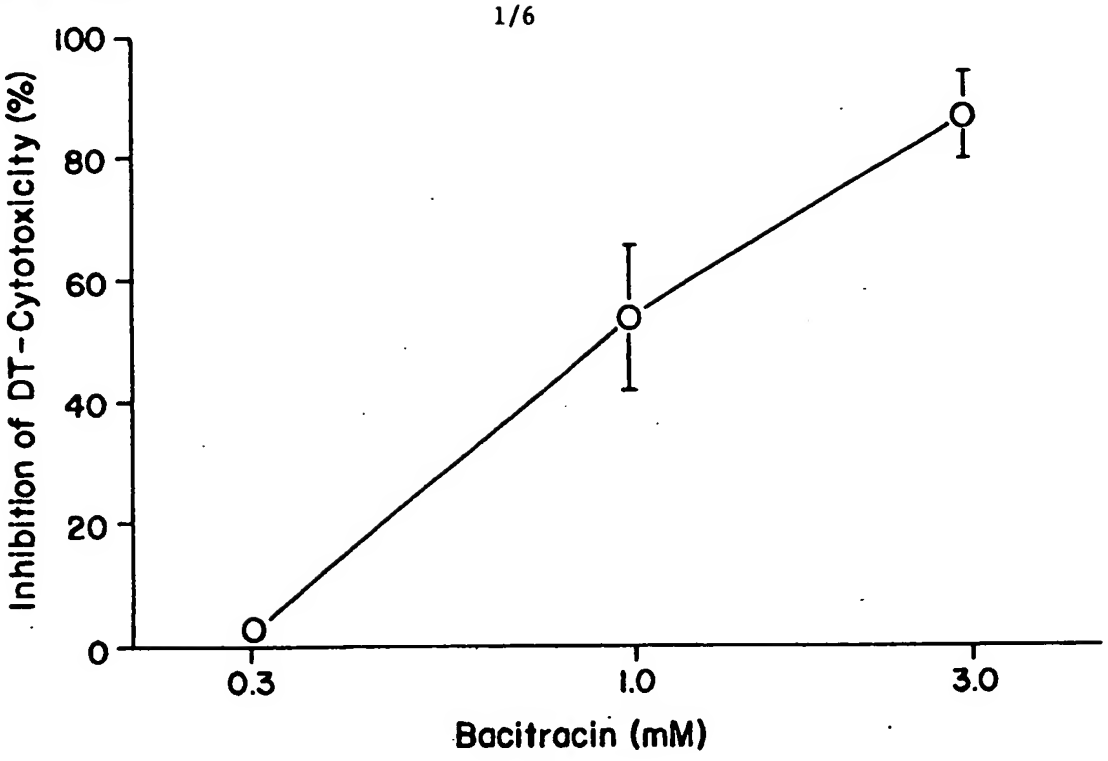
Inhibition of DT-Cytotoxicity (%)
100
80
60
40
20
0
0.3
1.0
3.0
Bacitracin (mM)

FIG. 1

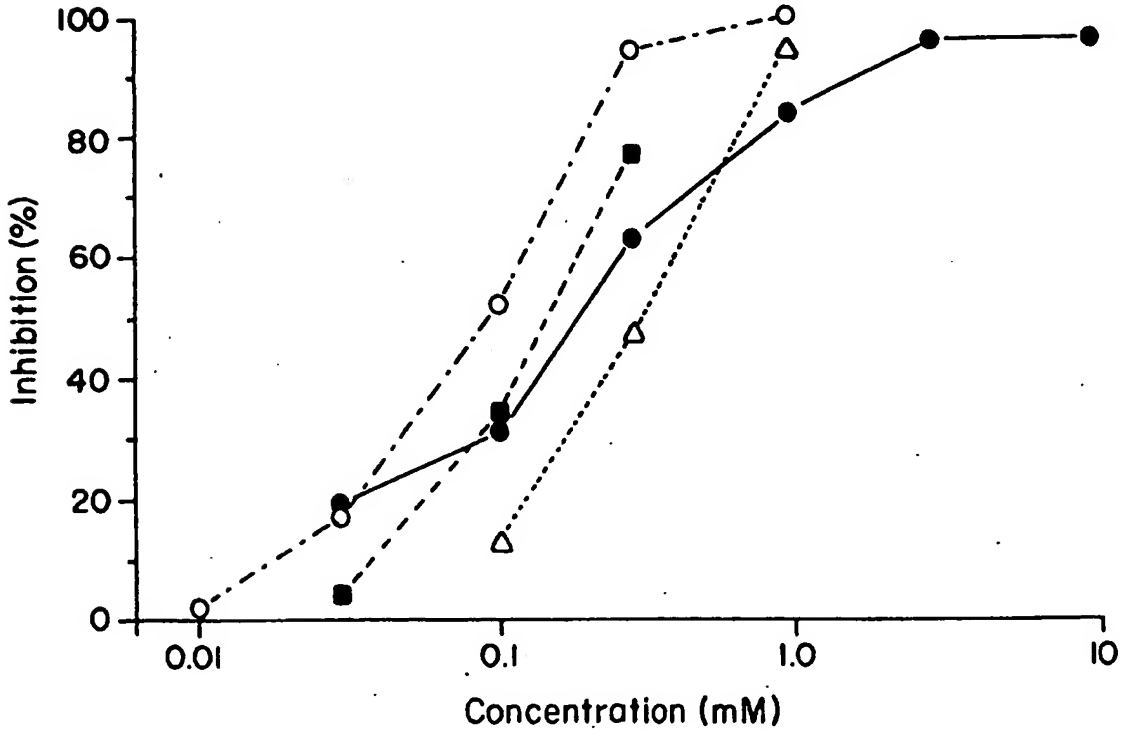
Inhibition (%)
100
80
60
40
20
0
0.01
0.1
1.0
10
Concentration (mM)

FIG. 2

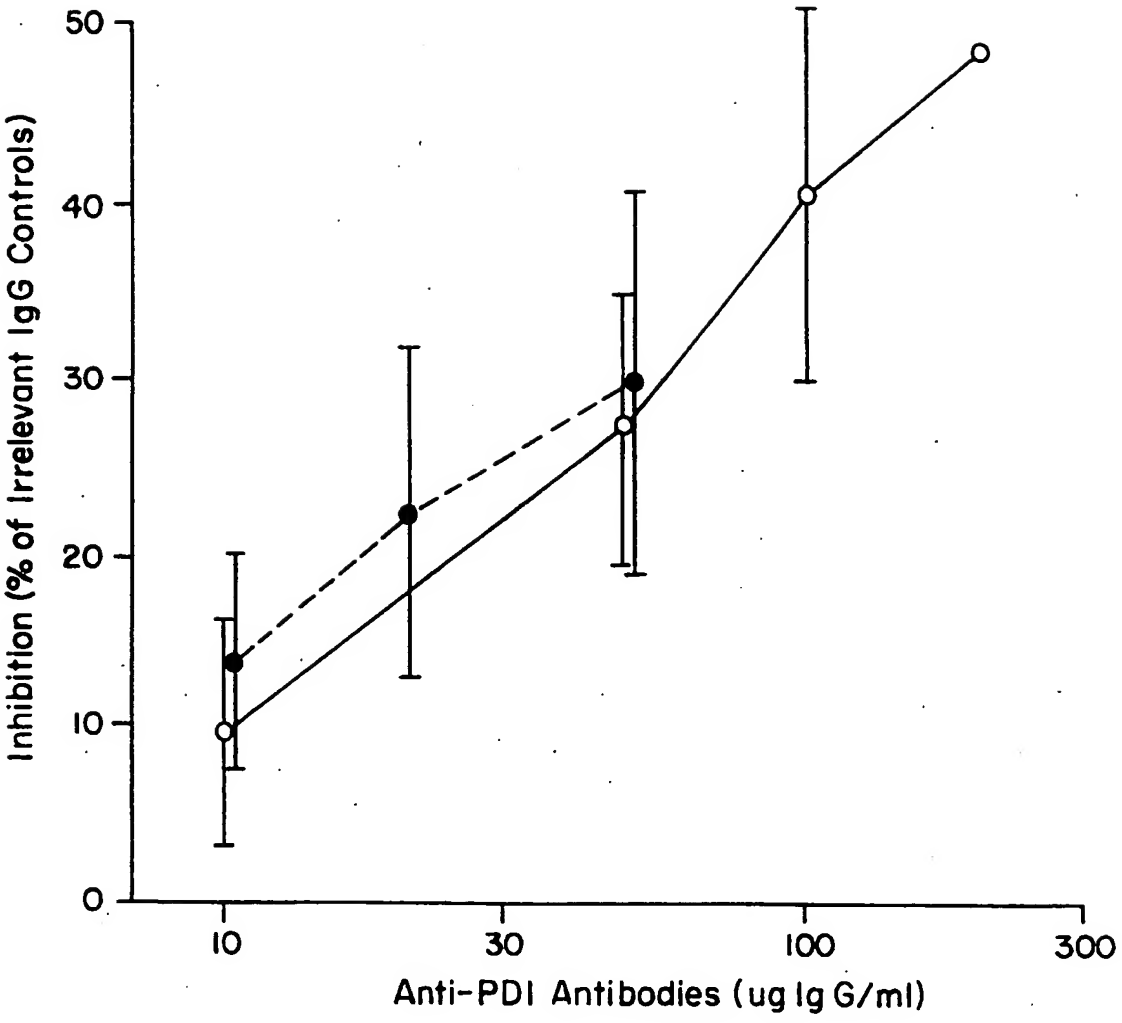
50
40
30
20
10
0
Inhibition (% of Irrelevant IgG Controls)
10
30
100
300
Anti-PDI Antibodies (ug Ig G/ml)

FIG. 3

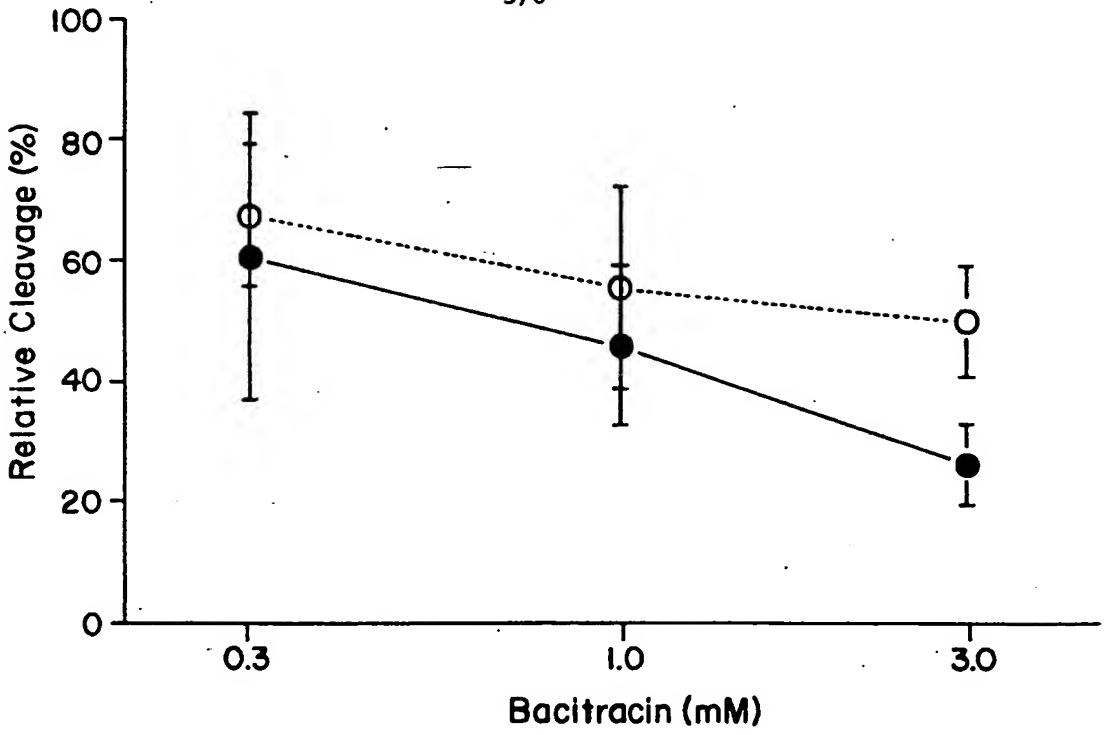
100
80
60
40
20
0
Relative Cleavage (%)
0.3
1.0
3.0
Bacitracin (mM)

FIG. 4

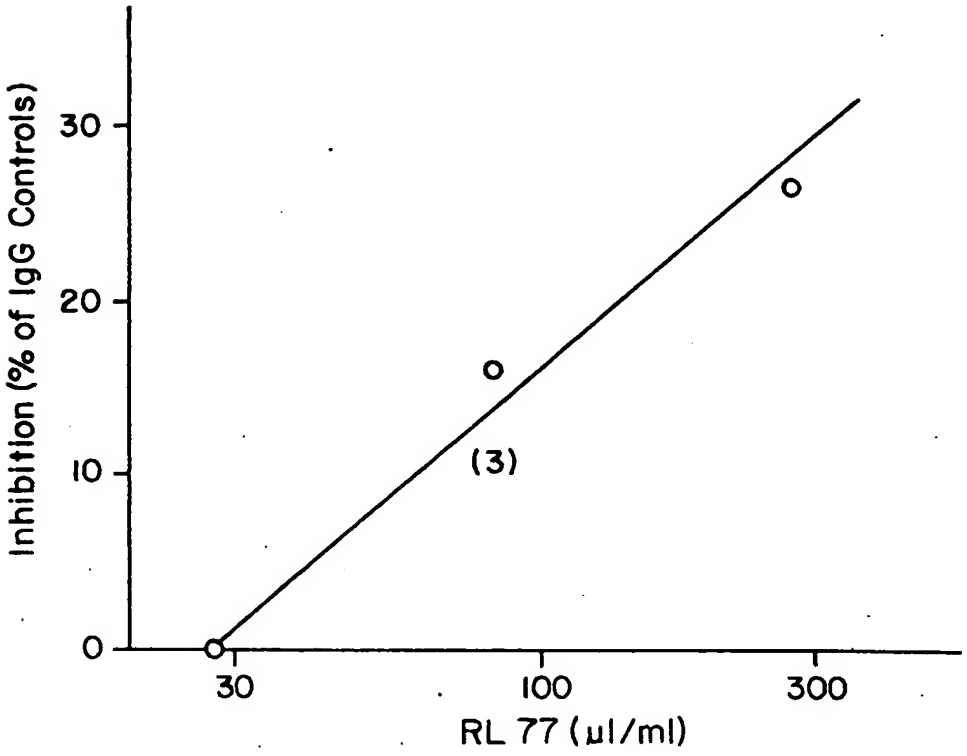
30
20
10
0
Inhibition (% of IgG Controls)
(3)
30
100
300
RL 77 (µl/ml)

FIG. 5

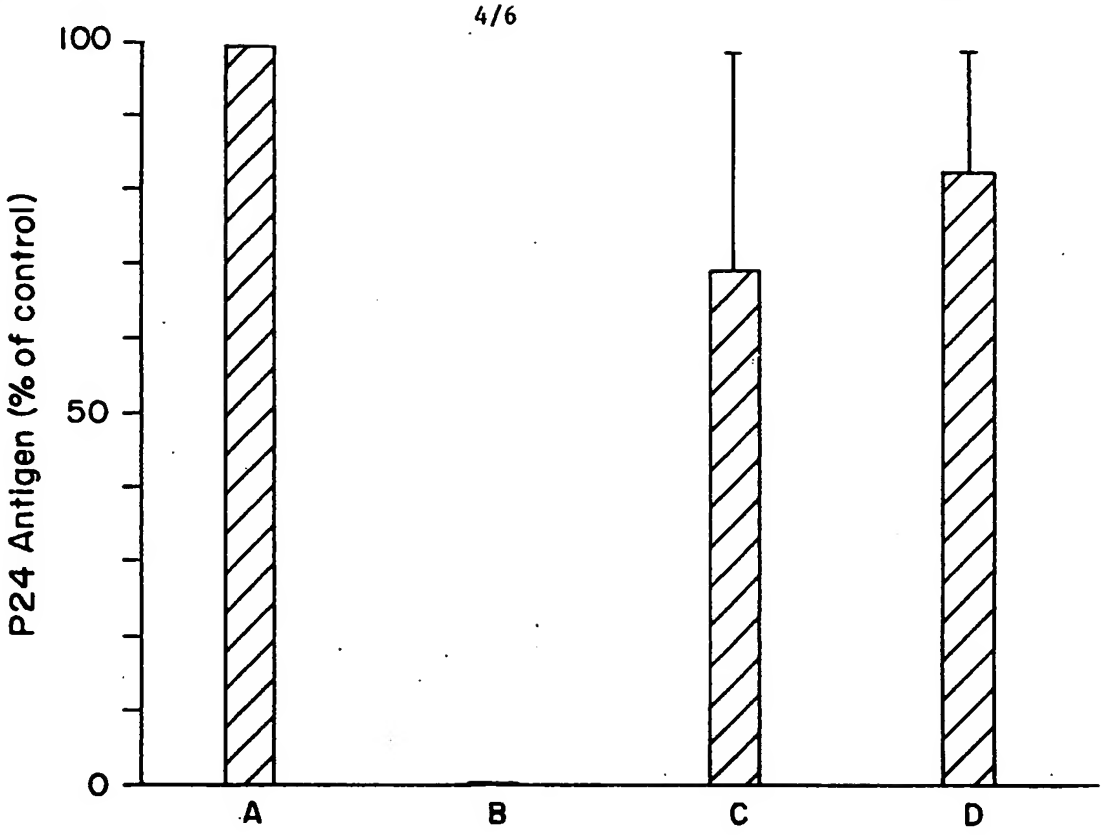
100
50
0
P24 Antigen (% of control)
A
B
C
D

FIG. 6

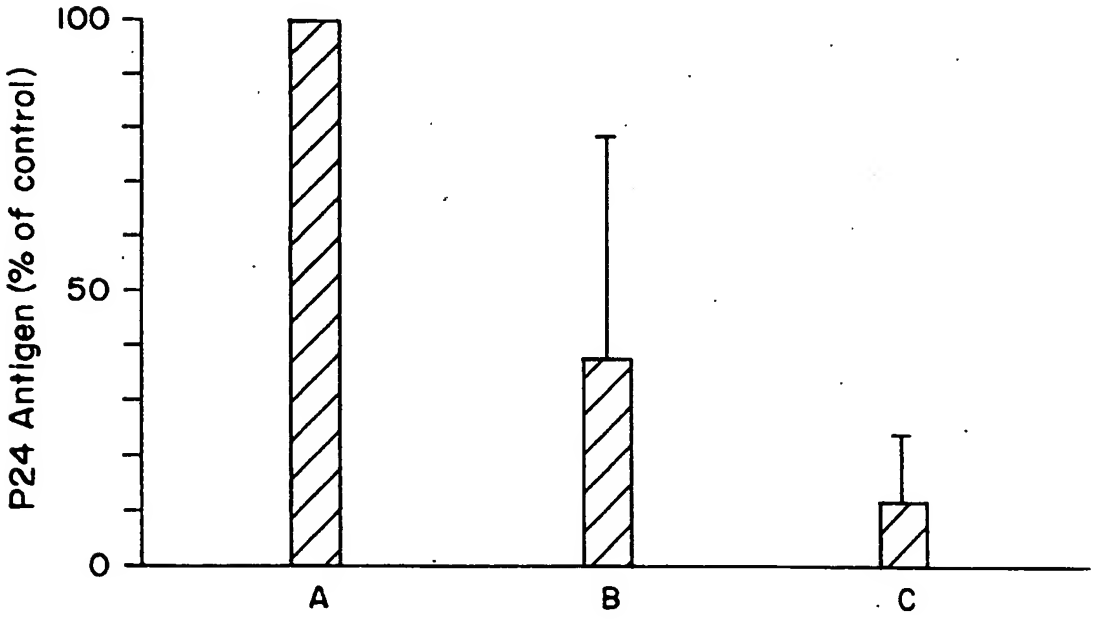
100
50
0
P24 Antigen (% of control)
A
B
C

FIG. 7

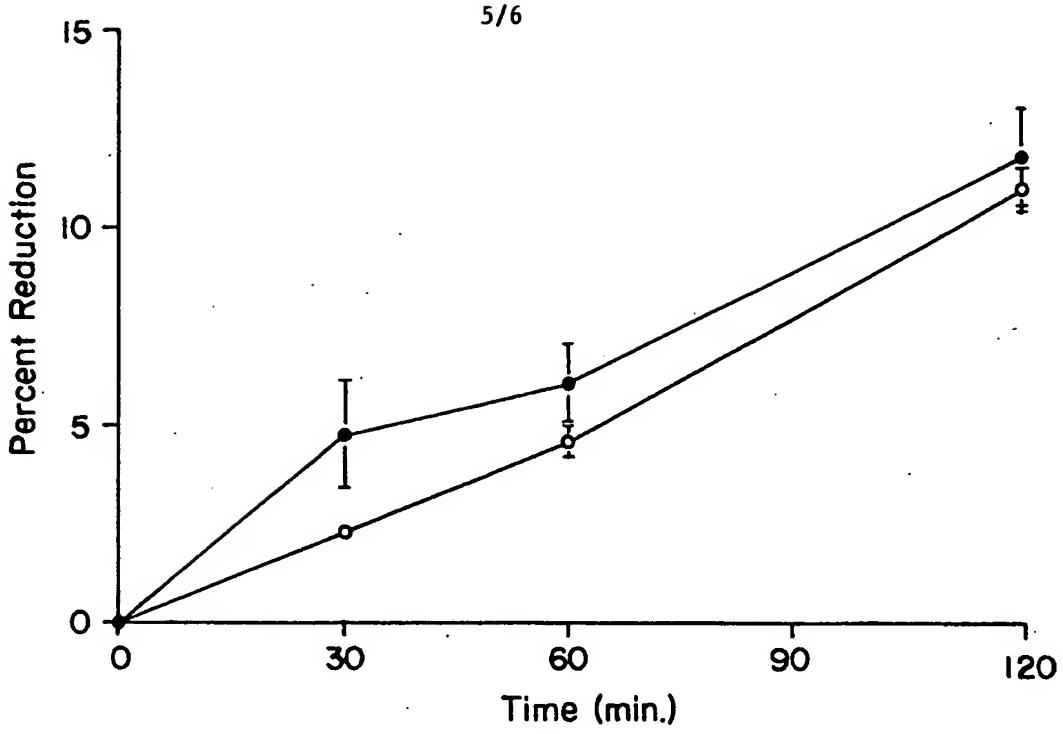
15
10
5
0
Percent Reduction
0
30
60
90
120
Time (min.)

FIG. 8

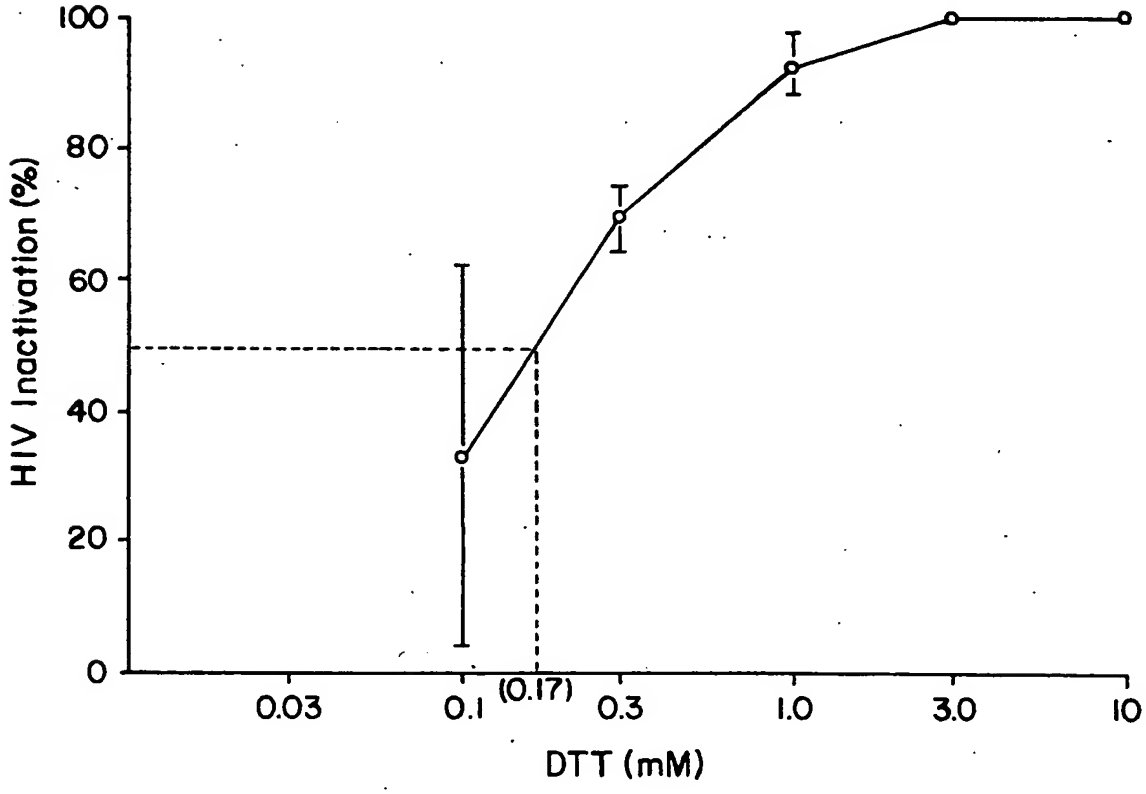
100
80
60
40
20
0
HIV Inactivation (%)
0.03
0.1
(0.17)
0.3
1.0
3.0
10
DTT (mM)

FIG. 9

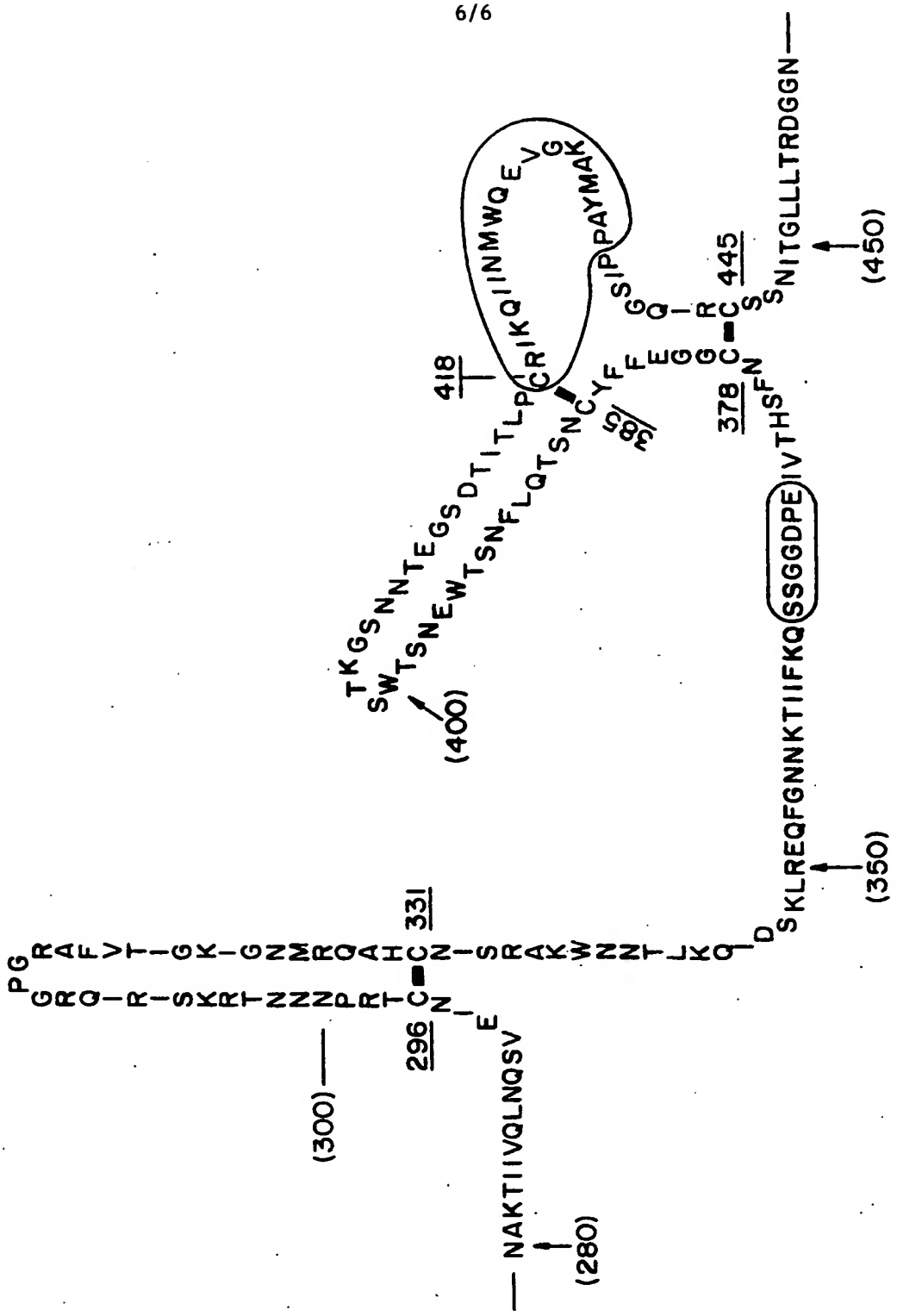
(280)
NAKTIIVQLNQSV
296
331
(300)
(350)
SKLREQFGNNKTIIFKQSSGGDPEIVTHSFN
378
445
SNITGLLLTRDGGN
(450)
418
(400)
TKGSNNTEGSDTITLPCRIKQIINMWQEVGKAMYAPPIEGQIRGCSGNWTFLGGTEENLYFQSC

FIG. 10